Novel platelet glycoprotein VI and CLEC-2 targeting strategies

Novel platelet glycoprotein VI and CLEC-2 targeting strategies: studies in humanized mouse models

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PhD thesis: Maastricht University, University of Würzburg
ISBN: 978-94-6469-297-6
Production: ProefschriftMaken
Cover Image: Maximilian Englert
Cover design: Stefano Navarro, Vera Van Beek

The research in this thesis was supported by a joint PhD scholarship of European Union´s Horizon 2020 research and innovation program under the Marie Sklodowska-Curie grant agreement TAPAS No. 766118

This thesis is submitted as a result of the joint doctorate program of Stefano Navarro at the Universities of Würzburg (Germany) and Maastricht (The Netherlands), for obtaining a double PhD degree. Admission to the joint doctorate program has been confirmed in the Individual Joint PhD Learning Agreement between the two universities and postgraduate researcher Stefano Navarro signed on 5 October 2021

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DISSERTATION

To obtain the degree of Doctor at Maastricht University and

the degree of Ph.D. at the University of Würzburg,

on the authority of the Rector Magnificus,

Prof. Dr. Pamela Habibović

and President Prof. Dr. Paul Pauli

in accordance with the decision of the Board of Deans

to be defended in public

on

Tuesday, 25th April 2023 at 13:00 hours

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Financial support for publication of this thesis by Stichting Hart Onderzoek Nederland (www.hartonderzoek.nu)is gratefully acknowledged.

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Neue Strategien zur Targeting von Glykoprotein-VI- und CLEC-2 inThrombozyten: Studien in humanisierten Mausmodellen

Thesis for a doctoral degree at the Graduate School of Life Sciences, Julius-Maximilians-Universität Würzburg,

Section Biomedicine

submitted by

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from Naples, Italy

Würzburg, 2023

Submitted on:

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Date of Public Defence: 25 April 2023 at 13:00 hours

Date of Receipt of Certificates:

Contents

Chapter 1

General Introduction

1. Physiology of platelets

Whereas the first observation of blood platelets has been attributed to different investigators,[1] a first description is commonly accredited to Max Schultze in 1865,[2] while shortly after that Giulio Bizzozero identified the role of platelets in thrombosis and hemostasis.[3,4] Platelets are currently known to be small, a-nucleated cell fragments derived from megakaryocytes, as precursor cells residing in the bone marrow.[3-5] In order to shed proplatelets, the megakaryocytes protrude parts of their plasma membrane together with cytoplasm and organelles into the blood vessels of the bone marrow. These proplatelets develop into immature or reticulated platelets, characterized by a relatively high RNA content. While circulating in the blood, the reticulated platelets progress into mature platelets with lower RNA levels.[6,7] While human platelets circulate for 7 to 10 days, mouse platelets are present for only 5 days.[8] Under normal conditions, human platelets have an average diameter of 1-2 µm and are present at a concentration of 150,000 to 400,000/µl. On the other hand, murine platelets are only 0.5 µm in size and circulate at 1,000,000 to 1,500,000/µl.[9]

Despite their small size, platelets are equipped with a complex system of cytoskeletal and membrane structures. The cytoskeleton ensures integrity and maintenance of the discoid shape of platelets; it also ensures a quick responsiveness to stimuli, *i.e*. by rapidly re-modeling cytoskeletal changes in the activated platelets (Figure 1).[5] The membrane structures of platelets include the open canalicular system, which is a tubular structure of the plasma membrane that invaginates into the cytosol, and allows platelet shape change upon activation; it also mediates granular membrane fusion as a prerequisite for secretion processes.[10]

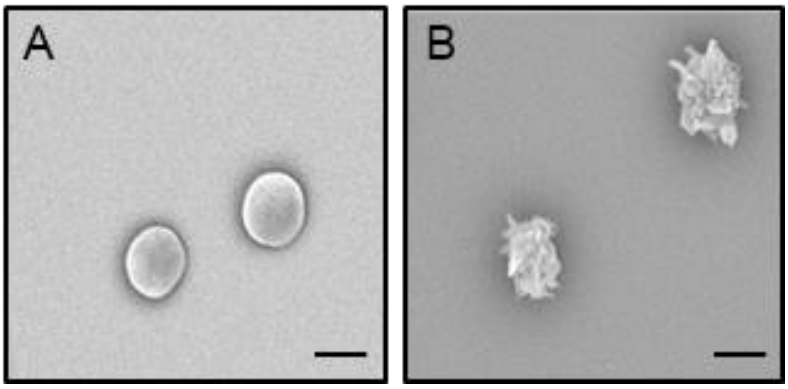

Figure 1. High resolution scanning electron micrographs of activated platelets. Scanning electron microscopy (SEM) of resting (A) and a filopodia-forming (B) murine platelets show the re-modeling capacity of the platelet cytoskeleton (scale bar 2 µm) after activation. SEM images were kindly provided by Dr. D. Stegner and Dr. S. Beck.

Platelet storage granules are present in two types, the α- and dense-granules. The first are loaded with multiple membrane-associated and soluble proteins, while the latter contain a non-protein cargo of the adenine nucleotides ADP and ATP, and also calcium ions and polyphosphates. Upon activation, platelets release these granular contents into the environment, which then act as secondary mediators for the propagation of thrombus formation and induce inflammatory responses.[10-12]

2. Platelet receptors

Platelets express on their surface a multitude of receptors, necessary for their interaction with the environment.[13] The most abundant receptor is the integrin αIIbβ3 (glycoprotein IIb/IIIa), which on a resting human platelet is present at 80,000-100,000 copies, yet with 20,000-40,000 more copies being available from storage granules and the open canalicular system.[14] Upon platelet activation, αIIbβ3 integrins undergo the transition from an inactive to an active conformation, which then allows the binding of fibrinogen, fibrin, von Willebrand factor (VWF) and other ligands. The integrin

conformation change thereby permits platelet-platelet interactions as well as platelet spreading and thrombus buildup.[15] Another abundant platelet receptor (~50,000 copies) is the GPIb-V-IX complex, composed of the protein chains GPIbα, GPIbβ, GPIX and GPV, which is important for a shear-dependent recognition of VWF and subsequent platelet adhesion.[16,17]

Among the numerous platelet receptors for activating agonists, most relevant are for this thesis are the three (hem)-ITAM receptors. These commonly act through the amino acid sequence YxxL/Ix$_{(6-8)}$YxxL/I, which forms the immunoreceptor tyrosine-based activation motif (ITAM), which is a recognition site for essential Src-family kinases.[18] The first ITAM-linked receptor, FcγRIIA (CD32), is a member of the immunoglobulin superfamily. It recognizes immune-complexes and is present on the surface of human platelets, but not on murine platelets.[17-19] The second receptor, glycoprotein VI (GPVI), is the major signaling receptor for collagen; its downstream signaling is dependent on its association with the co-receptor FcR γ-chain, which forms dimer carrying the ITAM-signaling sequences. The third, C-type lectin-like receptor-2 (CLEC-2), acts as a receptor for podoplanin, and forms a hem-ITAM receptor, since its cytoplasmic tail possesses only one ITAM domain (YxxL/I), implying that its dimerization is required for signal transmission.[18,20,21] Due to their relevance for this thesis, the two receptors, GPVI and CLEC-2, are discussed more in detail in the following paragraphs.

In addition, also several G-protein-coupled receptors (GPCR) for soluble agonists play a crucial role in the platelet activation process. Important for this thesis are the human protease-activated receptors PAR1 and PAR4 for thrombin, while mouse platelets express PAR3 instead of the PAR1 isoform.[22,23] Furthermore, platelets are equipped with the TP-type GPCR for thromboxane A_2 (TXA_2) and the $P2Y_1$ and

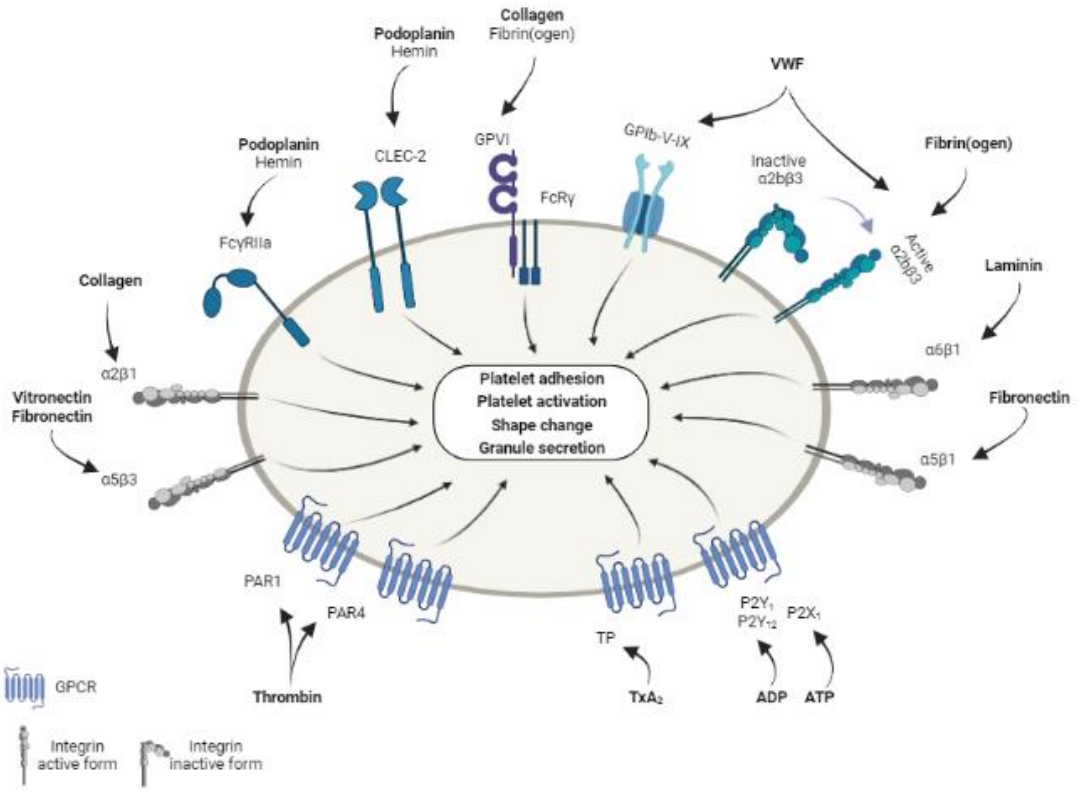

Figure 2. Schematic overview of the major platelet receptors involved in platelet activation and adhesion and their main ligands. For explanation, see text. Image created using Biorender.

$P2Y_{12}$ GPCR for ADP. These GPCR are involved in the promotion of platelet aggregation, via the secondary mediators TXA_2 and ADP. Their importance is illustrated by the fact that antagonism of TXA_2 and ADP actions is at the center of anti-platelet therapy for the secondary prevention of arterial thrombosis.[24-27] A schematic overview of these and other receptors on the platelet surface and of the key platelet responses is given in Figure 2.

3. Platelet activation and adhesion

Jointly with the coagulation system, platelets are fundamental for adequate hemostasis, thrombosis and vascular repair processes.[28,29] At the site of a vascular injury, platelets come into contact with extracellular matrix components, where they recognize collagen and VWF, with as a consequence the induction of platelet tethering,

adhesion, activation, aggregation and thrombus formation. The adhesion of platelets to the endothelial matrix is a complicated process, which starts under conditions of high shear by the interaction with collagen-bound VWF via the complex GPIb-V-IX, inducing initial tethering of flowing platelets. The VWF interaction with GPIb-V-IX is characterized by a rapid onset and a rapid dissociation rate, and is hence insufficient for stable platelet adhesion.[30] The main function of GPIb-V-IX is seen as a recruiting receptor for platelets at site of vascular damage, thus allowing platelet GPVI to interact with collagen and initiate platelet activation.

The platelet activation via GPVI is mediated by a protein tyrosine kinase cascade, and results in an increase in the cytosolic Ca^{2+} concentration, granule release, integrin αIIbβ3 activation via so-called inside-out signaling, and ultimately phosphatidylserine exposure.[31,32] The elevated cytosolic Ca^{2+} leads to arachidonate mobilization and its conversion into TXA_2 and to platelet degranulation, necessary for the release of second mediators like ADP. Both ADP and TXA_2 stimulate their GPCRs in order to induce full platelet activation.[33] The activation of αIIbβ3 and other integrins allows firm adhesion to several components present in the extracellular matrix and the blood plasma.[28] Well described integrins on the platelet surface are α2β1 and αIIbβ3. The receptor α2β1 plays an essential role in the adhesion to collagens,[34] whereas αIIbβ3 supports platelet adhesion to fibrinogen, fibrin and VWF. Binding of the bivalent fibrinogen molecule to two αIIbβ3 integrins allows the formation of bridges between platelets and the formation of platelets aggregates. The importance of αIIbβ3 is illustrated by the fact that its lack or dysfunction results in Glanzmann's thrombasthenia, which is a severe bleeding diathesis due to impaired platelet adhesion and abolished platelet aggregation.[35] Under flow conditions, integrin αIIbβ3 mediates thrombus formation and platelet adhesion to fibrin, so as to produce a stable clot.[36]

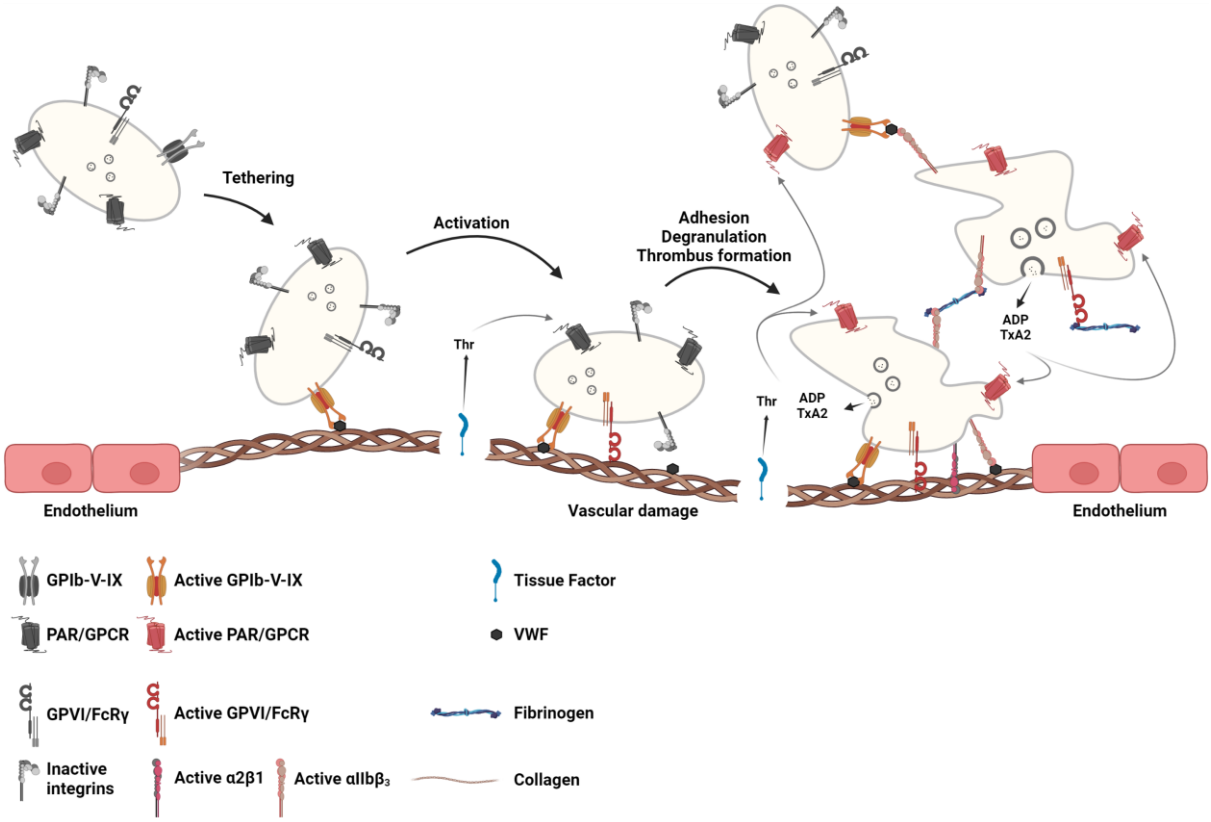

Figure 3. Sequences in platelet adhesion and activation under flow. At a site of vascular injury, the platelet receptor GPIb-V-IX binds to collagen-bound VWF, leading to the tethering of platelets at the subendothelial matrix. The subsequent binding of collagen to platelet GPVI induces granule release, leading to the release of ADP and TXA_2, both of which activate flowing platelets via their GPCR, thereby mediating the formation of platelet aggregates. Inactive receptors are indicated in grey, agonist-activated receptors are indicated in red. Platelet granules are depicted as grey circles. Abbreviation: Thr, thrombin. Image generated using Biorender.

Figure 3 shows the various stages of the collagen- and VWF-mediated adhesion process.

4. Coagulation cascade

In concert with platelet activation, the coagulation process starts on injured vessels by a cascade of plasma proteases, to mediate the formation of stable thrombi and fibrin clots. The coagulation cascade is commonly divided into two parts, the extrinsic and intrinsic pathways, which through different routes lead to the conversion of factor (F)X

into proteolytically active FXa. The extrinsic pathway is initiated by injury of a blood vessel. Tissue factor (FIII), present on vascular smooth muscle becomes de-encrypted and is exposed to the blood stream. It interacts with FVII(a), and subsequently with FX, inducing its activation to FXa.[37]

The second intrinsic coagulation pathway is initiated by FXII. The activation of FXII can occur on exposed collagen, and also by negatively charged surfaces such as collagen[38] and by polyphosphates secreted by platelets.[39] The formation of active FXIIa is dependent on kallikrein and high molecular weight kininogen (HMWK).[40,41] The FXIIa initiates a proteolytic cascade, leading to the activation of FXI, which subsequently activates FIX, thereby generating FIXa, needed for the high-rate activation of FX.

The generation of FXa marks the so-called common coagulation pathway.[42,43] Herein, FXa binds prothrombin (FII), FVa and Ca^{2+} ions, thereby forming the prothrombinase complex, responsible for the activation of prothrombin into thrombin. Similar to the high-rate FXa formation (by FVIIIa and FIXa), the generation of thrombin (by FVa and FXa) is strongly promoted by phosphatidylserine-exposing membranes, such as provided by highly activated platelets. Thrombin cleaves fibrinogen into fibrin monomers. With the help of FXIIIa, the fibrin monomers complex into large, cross-linked fibers, which form the basis of a blood clot.[40,42,44] A general scheme of these coagulation processes, as far as relevant for this thesis, is presented in Figure 4.

5. Interplay between platelets and the coagulation cascade

Both the coagulation process and the activation of platelets contribute to hemostasis and the generation of pathological thrombi.[45] Platelets participate in the coagulation process in several ways, although the expression of functional tissue factor on their

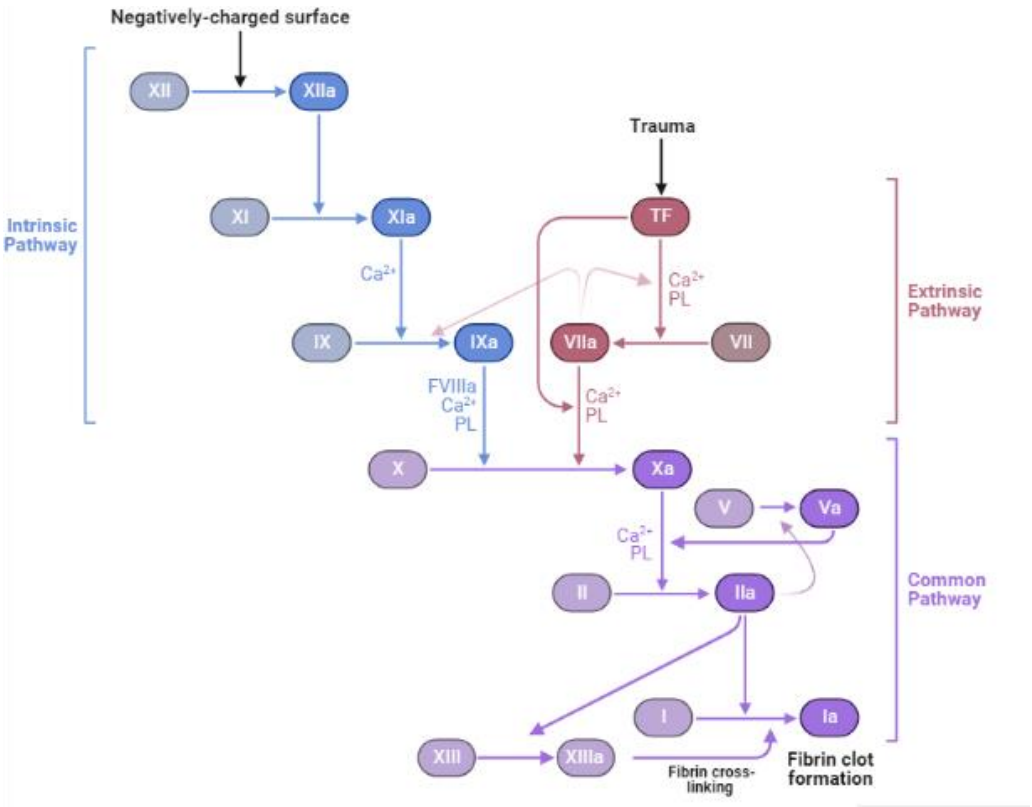

Figure 4. Simplified scheme of the coagulation cascades. The intrinsic pathway is represented in blue, the extrinsic in red, and the common pathway in purple. The scheme indicates main co-factors and secondary pathways of the cascade. Anticoagulant mediators are not indicated. *Abbreviations:* PL, phospholipids; TF, tissue factor. Image created using Biorender.

surface is controversial.[46] Thrombin that is produced as a result of the coagulation cascade cleaves and activates human platelets via the receptors PAR1 and PAR4, and mouse platelets via PAR3 and PAR4.[42,47] The coagulation end product, fibrin, as well as its precursor fibrinogen, support platelet-platelet interactions via the integrin αIIbβ3.[15] The process of fibrin degradation (fibrinolysis), less relevant for this thesis, is not introduced.

Recent evidence indicates that human GPVI (huGPVI) is able to recognize other ligands than only collagen. Thus, human platelet adhesion to the coagulation end product fibrin and also to fibrinogen leads to low-level spreading, activation and aggregation in a GPVI-dependent way.[48-50] Furthermore, in response to strong (GPVI)

agonists, the Ca^{2+}-dependent activation of platelets, via the ion/phospholipid channel anoctamin-6, leads to the exposure of a phosphatidyl-serine-containing surface as well as to swelling and balloon formation. The procoagulant phosphatidylserine membrane allows the assembly of coagulation factors at the platelet surface, thus supporting local FXa, thrombin and fibrin formation.[51]

The procoagulant platelets also show other pathophysiological properties, like the shedding of microparticles (extracellular vesicles) with a proinflammatory role.[52] An increased platelet procoagulant activity has been seen in patients with a risk of recurrent stroke or transient ischemic attacks.[53] Importantly, flow studies using blood from GPVI-deficient patients revealed a markedly lowered phosphatidylserine exposure, supporting that the formation of procoagulant platelets can be induced by GPVI activation.[54] Based on such findings, targeting of the procoagulant platelet population was proposed as a possible way of anti-thrombotic and anti-inflammatory therapy.[52,55]

6. The platelet CLEC-2 receptor

The type II transmembrane C-type lectin-like immune receptor, CLEC-2, of 32 kDa is encoded by the human gene *CLEC1B*. It has 6 exons and forms a protein of 229 amino acids. Structurally, CLEC-2 is composed of a C-type lectin-like domain, a stalk region, a single transmembrane helix and a short cytoplasmic tail.[56-58] In mouse, the ortholog *Clec1b* gene encodes for a similar but not identical protein (Section 10). The dimeric CLEC-2 on platelets induces downstream signaling responses.[59,60]

The best known ligand of CLEC-2 is podoplanin, which is a transmembrane protein expressed on several cell types, including fibroblastic reticular cells in the lymph nodes and on the lymphatic endothelium.[61] The binding of CLEC-2 to podoplanin is of

importance during development, since a lack of CLEC-2 on mouse platelets leads to blood-lymphatic mixing and premature death of the animals.[62] In addition, the interaction of podoplanin with CLEC-2 maintains the integrity of venular endothelial cells, and can function in cancer progression and in thrombo-inflammation.[63,64,65] A high expression of podoplanin has indeed been observed in squamous cell carcinomas.[66]

Alongside to podoplanin, also hemin[67] and galectin-9[68] can act as endogenous ligands for mouse and human CLEC-2. Besides these endogenous ligands, CLEC-2 can be activated by the snake venom rhodocytin, to result in rapid platelet activation.[69] In addition to the above-mentioned roles in thrombo-inflammation and tumor progression, recent literature also points to a role of CLEC-2 in cerebral venous thrombosis.[70] Altogether, this sparks the interest for further pharmacological studies to target CLEC-2 as a novel anti-platelet therapy for a range of diseases. As indicated in this thesis new mouse models promise to help in these investigations.

7. The platelet multiligand glycoprotein VI receptor

As a member of the Ig superfamily of receptors, huGPVI has a molecular weight of 62 kDa, of which 35 kDa are provided by the amino acids.[71,72] The *GP6* gene has 8 exons that encode for a protein of 319 amino acids. The protein structure consists of two Ig-like extracellular domains (D1 and D2), and furthermore a trans-membrane and a cytoplasmic region. The Ig-like domains are composed of structural β-sheets, and of these, the D1 is mainly involved in collagen binding.[73] Recent evidence further indicates that the D2 domain might play a role in GPVI dimerization.[74] The GPVI transmembrane domain allows the formation of a salt-bridge for connection to the co-receptor, FcR γ-chain. The cytoplasmic tail is indispensable for signal transduction.[21] The ortholog mouse *Gp6* gene produces a similar but not identical protein (see Section 10).

Importantly, human and mouse GPVI are only expressed by cells of the megakaryocyte-platelet lineage, with an estimated copy number of 4,000 to 9,600 per human platelet.[75] As a main receptor for the triple-helical collagens, GPVI binds to the collagen peptide composed of Gly-Pro-Hyp (GPO) repeats,[76] called collagen-related-peptide (CRP), especially in cross-linked form (CRP-XL). The snake venom convulxin is a non-endogenous, potent and multivalent ligand for GPVI, which quickly and strongly induces platelet activation, aggregation and thrombus formation. Similarities and differences between the human and mouse GPVI receptors are studied in this thesis.

As indicated above, fibrinogen and fibrin have been identified as novel ligands for the GPVI receptor.[49,50,77,78] In addition, several other ligands have been introduced, including α5-laminin,[79] fibronectin,[80] vitronectin,[81] galectin-9 and galactin-3,[68,82] and very recently the coagulation factor XIII.[83] However, the *in vivo* implications of the binding to these non-collagen ligands are still under investigation. The roles of huGPVI in health and disease are further discussed below.

8. ITAM signaling in platelets

Given that GPVI and CLEC-2 are both members of the ITAM signaling family, they share a similar downstream signaling cascade. The ITAM sequence ($YxxL/Ix_{(6-8)}YxxL/I$), described in Section 2, provides a unique set of tyrosines (Y) that can be phosphorylated. Nonetheless, some differences exist between the two receptors in signaling pathways. By itself, GPVI does not contain an ITAM sequence, meaning that for the translation of ligand information it requires hetero-dimerization with the co-receptor, FcR γ-chain. Of note, the FcR γ-chain is important not only for the GPVI signaling, but also for its expression on the platelet surface.[84] Upon ligand binding, the

Src-family kinases (SFKs) Fyn and Lyn bind to the proline-rich domain of GPVI via the SH3 domain, and then phosphorylate the tyrosines present on the ITAM sequence of the FcR γ-chain. The phosphorylated ITAM is recognized by the SH2 domains of Syk, which can be considered as the executive protein tyrosine kinase in ITAM signaling.

Once Syk is recruited, it starts to phosphorylate tyrosines on various adaptor proteins, such as LAT and SLP-76. The p85 subunit of phosphatidylinositol 3-kinase (PI 3-kinase) binds to a phosphorylated tyrosine of LAT, and then contributes to the signaling process. The adaptor protein SLP-76 is necessary for phospholipase Cγ2 (PLCγ2) recruitment. The tyrosine-phosphorylated PLC-γ2 recruited to the plasma membrane generates diacylglycerol (DAG), which activates protein kinase C (PKC), and furthermore inositol trisphosphate, which induces a cytoplasmic Ca^{2+} increase.[5,18,85-87]

Regarding the dimeric CLEC-2, with each protein chain possessing a single YxxL/I sequence (hemITAM), their binding to the SH2 domains of Syk is key for reciprocal phosphorylation and activation.[88] Once Syk has become phosphorylated and active, the downstream signaling pathway underneath CLEC-2 is similar to that of GPVI, although for CLEC-2 the functions of SFKs and Syk seem to be more cooperative, with Syk also participating to phosphorylation of the hem-ITAM. Moreover, CLEC-2 signaling is more dependent on second mediators and on cytoskeleton rearrangements, when compared to GPVI signaling.[5,89] The ITAM signaling cascade downstream CLEC-2 and GPVI is schematized in Figure 5.

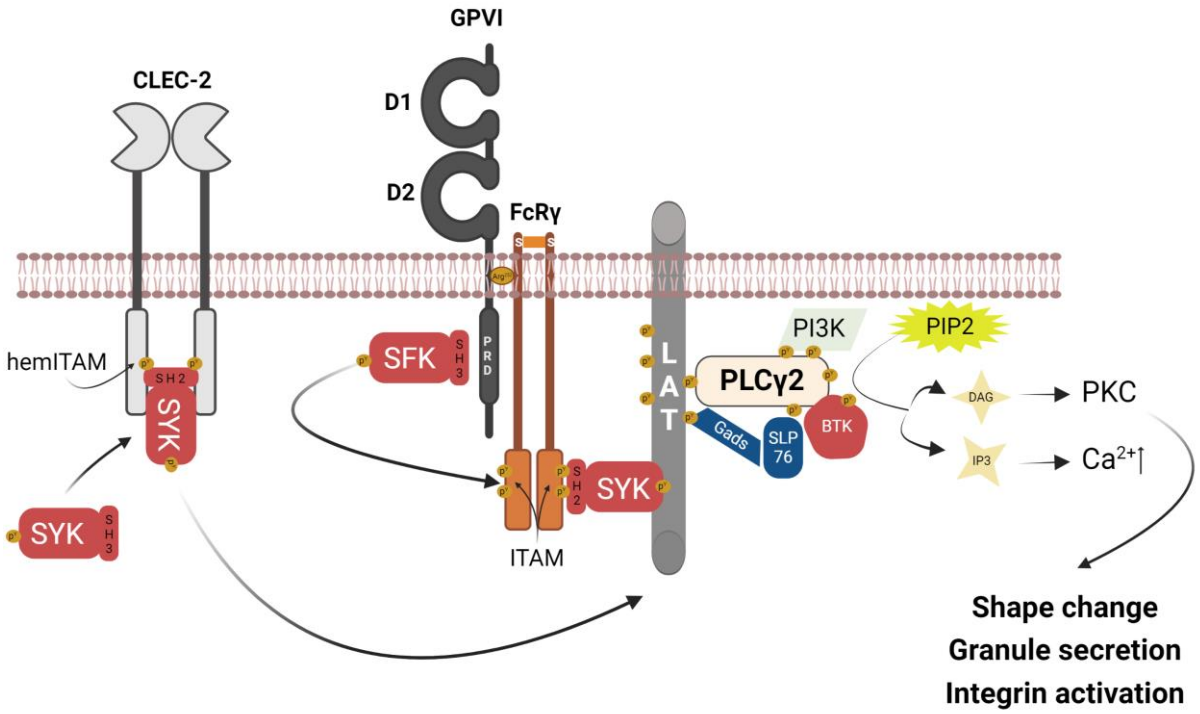

Figure 5. Representation of the platelet (hem)ITAM signaling cascade via GPVI and CLEC-2. Ligand binding and receptor clustering allows the tyrosines in the (hem)ITAM motif to become phosphorylated by the action of Src family kinases (SFK). The phosphorylation events lead to the recruitment and activation of the tyrosine kinase Syk, and thereby to initiation of the (hem)ITAM signaling cascade. Several scaffold proteins, adapters and effectors are recruited to the platelet membrane, forming a signalosome centered around the transmembrane protein LAT. Indicated are the subtle differences between GPVI and CLEC-2 concerning the initial signaling, although the major part of the signaling pathway is shared. Platelet shape change, granule secretion and integrin activation are common functional responses. Image created using Biorender.

9. Glycoprotein VI in health and disease

Platelet GPVI, as a key collagen receptor, is thought to play a major role in arterial thrombosis and a less crucial role in hemostasis.[90] A current concept is that collagen- and GPVI-dependent platelet activation is one of the first events after vascular injury to induce platelet degranulation, integrin activation and thrombus buildup.[5] The finding that patients lacking platelet GPVI due to a genetic mutation have no more than mild

bleeding symptoms has questioned the importance of the role of GPVI in primary hemostasis.[91,92] Similarly, mice lacking GPVI by genetic engineering or pharmacological intervention show only minor increases in bleeding time, as measured by tail injury.[93,94] This suggests that backup pathways exist to compensate for the loss of GPVI in hemostasis, for instance provided by tissue factor. Apart from its classical role in hemostasis, GPVI has been proposed to also contribute to other (patho)physiological processes. Activated GPVI supports the recruitment of leukocytes on an inflamed vessel wall, and it regulates vascular permeability.[95,96] Furthermore, GPVI-activated platelets can seal vascular breaches after neutrophil extravasation.[97]

Regarding the supposedly indispensable role of GPVI in arterial thrombosis, this has been confirmed in mice using multiple experimental thrombosis models including healthy or atherosclerotic arteries. Herein, the murine studies point to GPVI as a main effector for vaso-occlusive arterial thrombus generation. Thus, deficiency of GPVI or pharmacological targeting of GPVI resulted in a strong reduction of thrombus formation in all common *in vivo* models.[93,94,98] Furthermore, the collagen types I and III that are present in ruptured atherosclerotic plaques are effective ligands for GPVI.[99,100] There is also evidence that platelet GPVI is involved in the formation of platelet-leukocyte complexes in the context of venous thrombosis.[75]

Regarding vascular integrity, animal experiments, where GPVI was pharmacologically targeted or genetically depleted, have demonstrated an increase in tumor vascular leakage and a higher intra-tumor accumulation of chemotherapeutic drugs, thus reducing tumor growth.[101] Another study showed that the interaction of GPVI with galectin-3-expressing cancer cells favors tumor cell transmigration and metastasis.[82] In support of a role of mouse GPVI in inflammation, it appeared that a deficiency of GPVI reduced (inflammatory) atherosclerotic lesion size and neutrophil transmigration. Also in mice, the blockage of GPVI decreased inflammation in arthritic

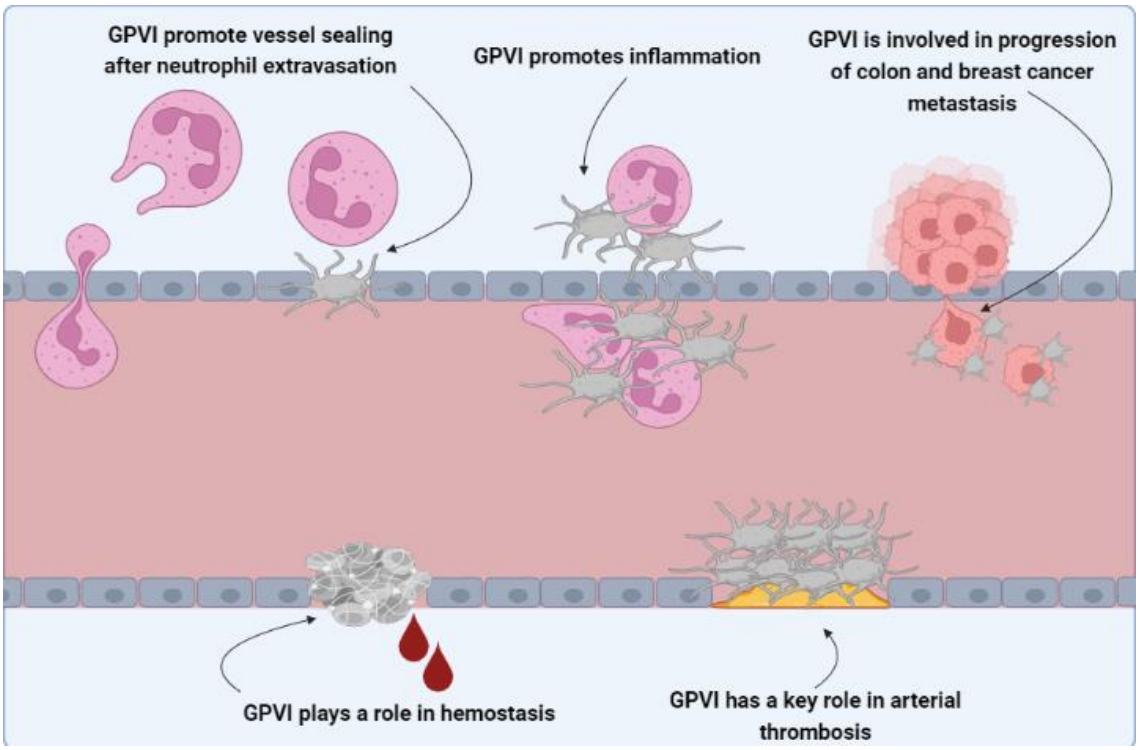

Figure 6. Overview of (patho)physiological processes involving platelet GPVI. Indicated are roles in hemostasis and thrombosis (lower part) and other processes such as vascular repair, inflammation and cancer (upper part). Neutrophils are represented as pink cells, platelets are grey. Image created using Biorender.

joints, possibly via a lower release of extracellular vesicles from platelets.[80,102]

Taken together, the literature points to multiple roles of platelets in general and GPVI in particular in hemostasis (though redundant), atherothrombosis, vascular integrity, tumor progression and inflammation. This makes GPVI an attractive target for antiplatelet strategies, aiming to attenuate arterial thrombosis with possible extensions to other clinical settings.

10. Humanized mouse models as tools to overcome inter-species GPVI and CLEC-2 differences

Genetic mouse models provide still an unsurpassed tool for scientific studies. Mouse and human share about 85% of their genome, which makes this animal suitable to

study many aspects of human physiology and disease.[103] However, many differences exist between the two species, with as an obvious example the difference in life-span. As mentioned above, mouse and human platelets are different in size, half-life and count. Typical inter-species differences are also present in the receptors studied in this thesis, GPVI and CLEC-2, despite the assumed high similarity in functions. The mouse *Gp6* gene is located on chromosome 7, while the human ortholog *GP6* is on chromosome 19. Mouse and human GPVI (mGPVI and huGPVI) share 67% of their nucleotide sequences and 64% of their amino acid sequences. The homology however is higher (78%) for the extracellular domain region of the protein. On the other hand, huGPVI has an intracellular domain that is 24 amino acids longer than that of mGPVI.[104]

These differences between mGPVI and huGPVI do raise the question if the receptor functions are identical. There are some indications for differences between the species. Studies report a difference in (activation-induced) GPVI shedding from the platelet surface, claiming that in mouse platelets GPVI is cleaved by both the ADAM10 and ADAM17 proteases, while in human platelets this job is mainly carried out by ADAM10.[105,106] Second, huGPVI but not mGPVI is able to support the activation and spreading of platelets on immobilized fibrinogen.[107] This may have implications for the precise roles of huGPVI and mGPVI in thrombus formation. For instance, huGPVI may have a more important role in supporting thrombus stability than mGPVI, under conditions where thrombin is absent.[108]

Also regarding CLEC-2 there are differences between the two species. In mouse, the *Clec1b* gene is located on chromosome 6, while the human ortholog *CLEC1B* is on chromosome 12.[109] The protein amino acid sequence shows an identity of 62% between human and mouse CLEC-2, with key sequences being more conserved.[110] Furthermore, although still under study, previous evidence showed a major difference in platelet expression levels (human 2,000-4000 copies, mouse

~40,000 copies).[20,60] Of note, the major signaling routes employed by the human and mouse receptors – for both GPVI and CLEC-2 – are considered to be well conserved.[110]

These inter-species differences may limit the unconditional use of mouse platelets to draw conclusions on the roles of human GPVI or CLEC-2. This is particularly relevant for the development of pharmacological agents to target the receptors, since even small differences in the receptor epitopes can influence the efficacy of drugs that are developed for the clinical setting. Humanized mouse lines - expressing human proteins – can be generated as tools to overcome these issues. Recently, a humanized mouse to test the functions and pharmacological targeting of huGPVI has already proven to be successful,[98] while no humanized mouse line for CLEC-2 has been reported so far. In this thesis, we used the huGPVI mouse and a newly generated huCLEC-2 mouse strain for the functional characterization of platelets and the evaluation of receptor-interfering tools.

11. Antiplatelet therapies

A common cause of pathological arterial vaso-occlusive thrombus formation is the rupture or erosion of an atherosclerotic plaque in the carotid or cardiac regions.[111] The disease-prone atheromatous plaques are composed of smooth muscle cells, macrophages, calcifications, fibrin and extracellular matrix components with collagens.[99,112,113] Upon rupture, such plaques expose a variety of platelet-activating agents as well as procoagulant tissue factor, thereby leading to the rapid formation of pathological thrombi. Occlusive thrombus formation upon erupted or eroded plaques is a main cause of ischemia leading to myocardial infarction or stroke,[114] as the formed thrombi and their emboli will occlude smaller vessels downstream in the vasculature.

Antiplatelet therapy plays a critical role in the secondary prevention or

attenuation of arterial ischemic events.[115] Although earlier trials with antiplatelet drugs in venous thrombosis have not been very successful, there is recent evidence that platelets do contribute to venous thrombosis.[114,116-118] Additive antiplatelet therapy has also been of some success in inflammation[119] and cancer metastasis.[101,120]

Acetylsalicylic acid (aspirin) has for long been a gold standard in antiplatelet therapy.[121] Aspirin irreversibly acetylates and blocks cyclooxygenases (COX), and it thus suppresses TXA_2 synthesis, thereby eliminating a secondary mediator of platelet activation.[122] Aspirin blocks not only the constitutive isoform COX-1, but also the inducible isoform COX-2, which is upregulated in various cells in inflammation.[123,124]

The more recent use of anti-$P2Y_{12}$ inhibitors has improved the clinical outcomes, for instance in patients with acute coronary syndromes,[125] either as monotherapy[126] or as dual therapy in combination with aspirin.[127] The two common inhibitors, clopidogrel and prasugrel, are prodrugs producing an irreversible inhibitor of the platelet $P2Y_{12}$ receptors.[128] The related drug ticagrelor reversibly inhibits $P2Y_{12}$, and was found to have a higher efficacy after acute coronary syndrome than clopidogrel. The blockade of $P2Y_{12}$ suppresses most of the ADP-dependent secondary events in platelet activation, although leaving the $P2Y_1$ signaling unchanged.[126,129] This explains why dual antiplatelet therapy is so successful in reducing thrombotic events.[127,130,131]

Integrin αIIbβ3 blockers add to the current list of antiplatelet therapeutics.[132] The αIIbβ3 inhibitors, eptifibatide, tirofiban, abciximab and RUC-4,[133-135] act as intravenous antiplatelet agents that block the binding of fibrinogen and VWF.[136] Their use is mostly limited to patients undergoing percutaneous coronary interventions after an acute coronary syndrome.[137]

Given that the common antiplatelet drugs carry a risk of bleeding,[138] pharmaceutical research is moving towards the development of drugs with minimal or no bleeding side effects. Interesting targets are PAR1 and PAR4, the thrombin

receptors present on human platelets and vascular cells.[139] The first PAR1 inhibitors, vorapaxar and atopaxar, demonstrated clear *in vitro* antiplatelet effects.[140] Clinical trials for vorapaxar showed a benefit for the secondary prevention of atherothrombotic ischemic events,[141] whereas atopaxar had off-target effects such as an altered heart rate and abnormal liver function.[142] In monkey models, the selective PAR4 inhibitors, BMS-986120 and BMS-986141, demonstrated antithrombotic efficacy with a minimal increase in bleeding time, when compared to clopidogrel; these compounds are now further developed.[143] In addition, CLEC-2 is on the list of targetable receptors due to its role in thrombo-inflammation, cancer and metastasis. However, no anti-CLEC-2 drug is currently available for clinical use.[64,65,70,144] An overview of the common anti-platelet therapeutics is given in Figure 7.

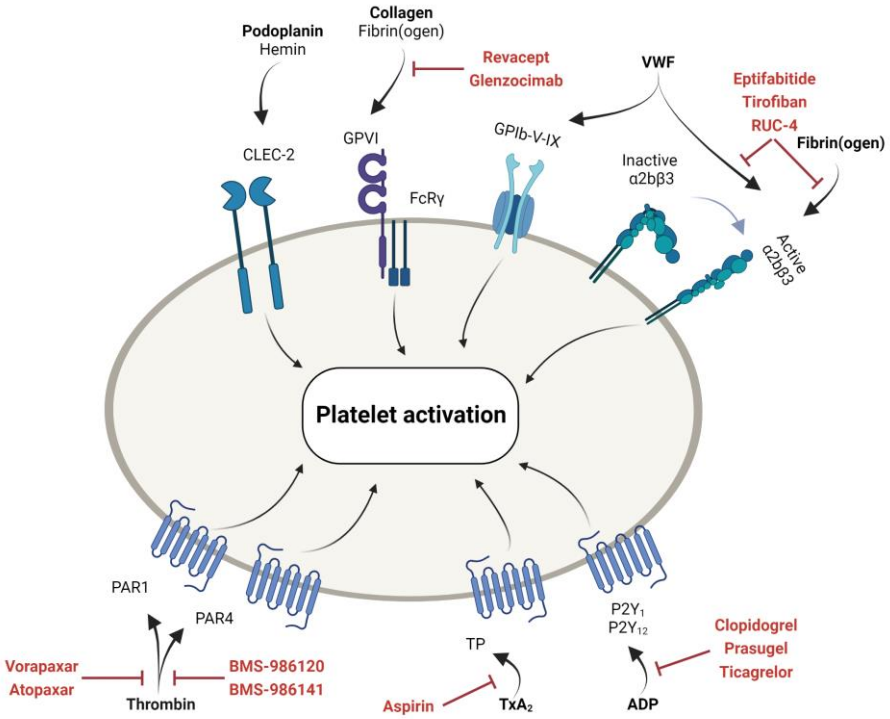

Figure 7. Cartooned overview of antiplatelet drugs (red) used in the clinic or under investigation in clinical trials. Arrows indicate activation of the receptors, whereas inhibitory line indicate antagonizing action. For explanation, see text. Imaged created using Biorender

12. Targeting platelet GPVI

For many years, GPVI has been proposed as a target for antiplatelet agents.[145] Several compounds have been investigated for this purpose. Losartan is an angiotensin II receptor antagonist that was thought to inhibit GPVI by blockade of its clustering.[146,147] Honokiol is a natural bioactive molecule used in traditional Chinese medicine with anti-oxidant, anti-inflammatory, anti-depressant, anti-cancer and anti-neurodegenerative activity. This compound was also considered to inhibit GPVI activation.[146,148] However, both drugs, losartan and honokiol, appeared not to act as specific GPVI inhibitors, since they also affected FcγRIIA and CLEC-2 signaling in platelets.[146]

Revacept is a dimeric GPVI ectodomain construct fused to the Fc domain of human IgG1. It functions as a competitive GPVI inhibitor by preventing platelet binding to collagen, reducing thrombus formation, but without effect on bleeding time.[149,150] After favorable results in a phase I study,[149] Revacept was shown to not reduce the incidence of myocardial injury in patients with stable ischemic heart disease.[150]

An approach to specifically target GPVI is to use blocking monoclonal antibodies. The first reported antibody inhibiting GPVI was JAQ1, a rat IgG targeting mouse GPVI.[87] In mice, *in vivo* administration of the JAQ1 antibody resulted in GPVI depletion through an Fc-dependent mechanism in the liver.[153] The injected mice adopted a sustained GPVI-knockout-like phenotype with long-term antithrombotic protection.[93] Interestingly, in several immune thrombocytopenia patients[145,154] and for human platelets infused into mice,[155] it appeared that the IgG-mediated GPVI depletion mechanism also applies to human platelets.

In addition, Fab fragments of anti-GPVI antibodies are being used to block GPVI in mice,[101,156,157] as well as in non-human primates and human subjects.[156,157] Currently, the first antibody-derived GPVI inhibitors are about to enter the clinic. A

phase II study with the monovalent antibody ACT017 (glenzocimab, a modification of the 9O12 Fab) indicated high efficacy as add-on therapy to tissue plasminogen activator (tPA) in the acute phase of ischemic stroke.[157,158] Among several other approaches to target GPVI, mostly with a deduced antithrombotic effect, only glenzocimab has given positive results in clinical trials.[156,158] Similarly, biochemical characterization of the murine version of ACT017 (modified 9O12 Fab) showed its binding to circulating platelets, and a half-life of 2.5 hours.[98,156,157] The humanized monovalent antibody ACT017 on the other hand, suppressed the GPVI-induced platelet aggregation for only several hours after injection,[156] hence giving space for improvement. To further address this, the generation and characterization of new GPVI inhibitors is still ongoing and is studied in the present thesis.

Recently, a nanobody (small-sized cameloid antibody) approach to target GPVI has become available, demonstrating promising *in vitro* effects on plaque-induced GPVI clustering, GPVI signaling and thrombus formation under flow.[159] However, the small size of nanobodies of 12-14 kDa lies below the renal filtration cut-off of 30-50 kDa.[160,161] Accordingly, nanobodies can be easily cleared from the blood stream via renal filtration after *in vivo* injection, strongly reducing their pharmacologically efficacy.[162,159] To sum up, platelet GPVI is a promising therapeutic target that serves high attention to obtain improved tools for treatment options.

13. Aims and outline of this thesis

The hypothesis of the work presented in this thesis is that the targeted modulation of ligand-induced GPVI or CLEC-2 activation provides anti-thrombotic protection with minimal effects on normal hemostasis. The aims of my studies accordingly are to fully characterize CRISPR-Cas9-generated GPVI and CLEC-2 humanized mouse lines, and

subsequently using established *in vitro* and *in vivo* assays to study platelet functions. The characterized mouse lines are then used for the validation of known and novel inhibitors to target the ITAM receptors. The current **Chapter 1** provides general background of the topics and platelet functions studied in this thesis. **Chapter 2** focuses on the characterization of a mouse line humanized for GPVI (*hGP6*$^{tg/tg}$), and its use to assess effects of the anti-GPVI monoclonal antibody JAQ1 on receptor function. **Chapter 3** unravels the time-dependency of pathways involved in GPVI-dependent thrombus formation under coagulant conditions. The chapter also considers the effects of the novel anti-GPVI inhibiting EMF-1 Fab in affecting thrombus development in the presence of tissue factor-induced coagulation. **Chapter 4** describes a comprehensive study on the depletion of human GPVI (huGPVI) as a safe and effective anti-thrombotic strategy, and investigates the efficacy of treatment of *hGP6*$^{tg/tg}$ animals using the EMF-1 IgG. **Chapter 5** concerns the characterization of a novel mouse model humanized for CLEC-2 and the development of a new monoclonal anti-human CLEC-2 antibody, HEL1, for *in vivo* testing. This chapter also illustrates the power of the humanized CLEC-2 mouse model for evaluating novel therapeutics *in vivo*. Finally, **Chapter 6** critically discusses the work presented in the thesis in the context of the current literature.

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Chapter 2

Targeting of a Conserved Epitope in Mouse and Human GPVI Differently Affects Receptor Function

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Int. J. Mol. Sci. 2022, 23(15), 8610

I performed the experiments, analyzed the data, and wrote the manuscript. A.S. performed experiments. J.W.M.H. and M.J.E.K. provided supervision, analyzed data and did proofreading of the manuscript. B.N. and D.S. designed research, analyzed data, provided expert supervision and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Abstract

Glycoprotein (GP)VI is the major platelet collagen receptor and a promising anti-thrombotic target. This was first demonstrated in mice using the rat monoclonal antibody JAQ1, which completely blocks the collagen-related peptide (CRP)-binding site on mouse GPVI and efficiently inhibits mouse platelet adhesion, activation and aggregation on collagen. Here, we show for the first time that JAQ1 cross-reacts with human GPVI (huGPVI), but not with GPVI in other tested species, including rat, rabbit, guinea pig, swine, and dog. We further demonstrate that JAQ1 differently modulates mouse and human GPVI function. Similar to its effects on mouse GPVI (mGPVI), JAQ1 inhibits CRP-induced activation in human platelets, whereas, in stark contrast to mouse GPVI, it does not inhibit the adhesion, activation or aggregate formation of human platelets on collagen, but causes instead an increased response. This effect was also seen with platelets from newly generated human GPVI knock-in mice (hGP6tg/tg). These results indicate that the binding of JAQ1 to a structurally conserved epitope in GPVI differently affects its function in human and mouse platelets.

Keywords: glycoprotein VI; JAQ1; platelet receptors; platelet activation; platelet inhibition

Introduction

Platelets are small, a-nucleated blood cells produced by bone marrow resident megakaryocytes, that have key roles in hemostasis and thrombosis[1, 2] At sites of vascular injury, platelets recognize and bind to specific ligands on the exposed extracellular matrix, become activated and aggregate to form a hemostatic plug that seals the vessel and prevents excessive blood loss. However, in pathological conditions intravascular platelet activation can precipitate the formation of vessel-occluding thrombi, leading to ischemic disease states such as stroke or myocardial infarction.[3,4] Therefore, anti-platelet drugs have become indispensable therapeutics to efficiently prevent or treat arterial thrombosis, but they carry an inherent risk of bleeding, most obvious in multimorbid patients requiring dual platelet inhibition or combined anticoagulation.[5] Among the major platelet receptors, glycoprotein (GP)VI has emerged as a promising therapeutic target, as its functional inhibition or genetic deletion provides protection from arterial thrombus formation *in vivo*, while only minimally affecting hemostasis.[6] GPVI is the main signaling receptor for collagen and expressed exclusively on platelets and megakaryocytes. GPVI is associated with the FcRγ (Fc receptor γ)-chain, which is responsible for the signaling via its immuno-receptor-tyrosine-based-activation-motif (ITAM). Besides collagen, several additional physiological agonists have been identified in recent years. These include fibrinogen, fibrillar fibrin[7-10] and fibronectin,[11] the basement membrane protein nidogen-1[12] and laminins.[13] These ligands are likely to – at least in part – contribute to the role of GPVI in pathophysiological processes beyond thrombosis, such as ischemia-reperfusion injury,[14] sepsis,[15] cancer and metastasis,[16,17] and venous thrombosis.[18] Collectively, these studies highlight the potential benefit of efficient anti-GPVI agents. In fact, first inhibitors of GPVI are entering the clinic. The GPVI-blocking Fab (ACT017,

glenzocimab) is assessed in the context of acute ischemic stroke.[19-21] In transgenic mice carrying the human *GP6* gene, glenzocimab was found to be effective in thrombus suppression, without impacting GPVI-dependent inflammatory hemostasis.[22] The first studies on the (patho)physiological function and in vivo relevance of GPVI were performed in mice and capitalized on the first reported anti-GPVI monoclonal antibody (mAb), JAQ1 (rat IgG2a).[23,24] It was shown that JAQ1 completely blocks collagen-related-peptide (CRP)-induced activation of mouse platelets and virtually abolishes mouse platelet adhesion, activation and aggregate formation on collagen.[6,25] Notably, a possible interaction of JAQ1 with human GPVI (huGPVI) has not been studied. Mouse and huGPVI share ∼67.3% of their nucleotide sequence and ∼64.4% of the amino-acid sequence, with huGPVI having an intracellular domain that is 24 amino acids longer than that of mGPVI.[26] In addition, also the extracellular domains of the receptor differ between the two species, best documented by the ability of huGPVI, but not mGPVI, to support platelet spreading on fibrinogen.[27] As this raises the question whether a specific anti-mGPVI antibody could bind huGPVI and thereby modulate its function in a comparable manner,[28] we assessed the effects of JAQ1 on huGPVI. Here, we show that JAQ1 recognizes a conformational epitope on huGPVI and efficiently activates human platelets upon cross-linking by a secondary antibody. Similar to its effect on mGPVI, JAQ1 inhibited human platelet activation by CRP, but in stark contrast to mouse platelets it did not inhibit, but rather promote adhesion, activation and aggregate formation of human platelets on collagen. These differential effects of JAQ1 on huGPVI were confirmed in platelets from a newly generated mouse line expressing huGPVI instead of mGPVI

Results

Anti-mouse GPVI monoclonal antibody JAQ1 binds human GPVI and modulates receptor function

JAQ1 (rat IgG2a) is the first anti-GPVI mAb reported in the literature and was initially raised against mouse GPVI (mGPVI).[24] JAQ1 completely blocks the major collagen binding site / CRP binding site in mGPVI[6] resulting in profound inhibition of platelet adhesion, activation and aggregate formation on collagen *in vitro.*[6,24,29] To assess potential cross-reactivity of JAQ1 with GPVI in other species, we assessed binding of JAQ1-FITC to platelets in freshly prepared diluted heparinized blood by flow cytometry. In agreement with previous descriptions, JAQ1-FITC robustly bound to *WT*, but not *Gp6*$^{-/-}$ mouse platelets (Figure 1A). In addition, we observed no binding to platelets from closely related species such as rat, rabbit, guinea pig, swine or dog (Table 1). Remarkably, however, JAQ1-FITC robustly bound to human platelets. (Figure 1A, Table 1). Next, we assessed whether JAQ1 binding would be affected by pre-incubation of human platelets with different established anti-huGPVI mAbs. Indeed, JAQ1-FITC binding was reduced by ~66% after pre-incubation with the function blocking anti-huGPVI mAb EMF-1,[30] but only partially by the non-function blocking EMF-2 (~26%) (Figure 1B). Subsequently, we tested whether JAQ1 recognizes huGPVI in a Western blot analysis of human platelet lysate. However, while JAQ1 efficiently detected mouse GPVI (mGPVI) no band in human platelet lysates was observed, indicating that the epitope on huGPVI is conformation sensitive (Figure 1C).

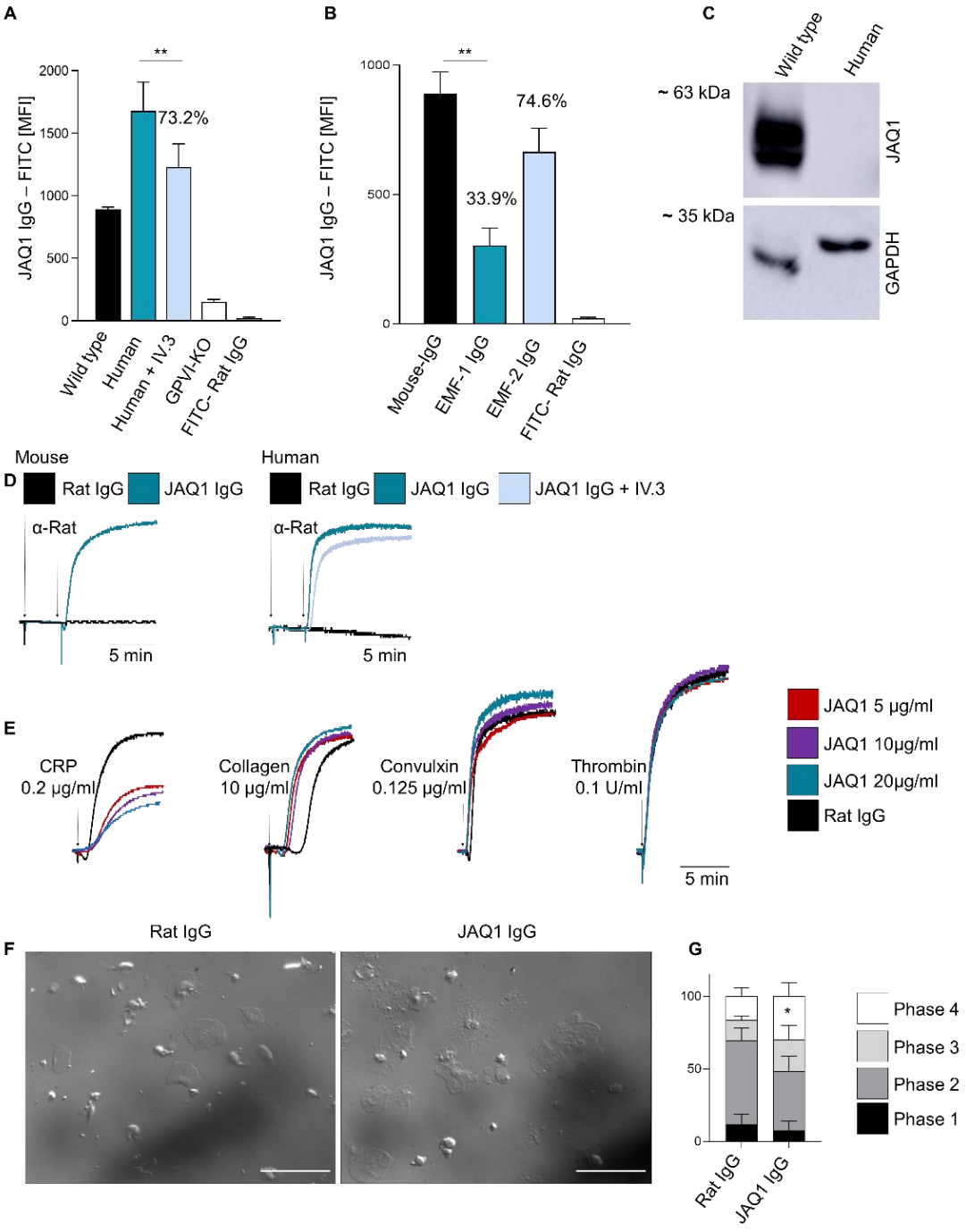
Table 1. Flow cytometric analysis of JAQ1 binding to the platelet surface of different species. The data are reported as MFI of JAQ1-FITC (JAQ1 MFI) and as percentage respect to mouse MFI (JAQ1%). Mean platelet volume (MPV) of the different species are reported in femtoliter (MPV). "Negative" indicates MFI values comparable to isotype IgG-FITC control values. Data are reported as means ± SD.

Species	JAQ1 MFI	MPV (fl)	JAQ1 %	MPV Reference
Mouse	995 ± 24.5	5.5-6	100%	[31]
Rat	Negative	4.4-6.9	-	[32]
Rabbit	Negative	5.55-6.35	-	[33]
Guinea pig	Negative	7.1-8.2	-	[34]
Swine	Negative	8.4-9.75	-	[35]
Dog	Negative	8-12	-	[36]
Human	1296 ± 47.7	9.4-12-3	130%	[37]

Next, we assessed the effects of JAQ1 on human platelet aggregation. Cross-linking of JAQ1 with a secondary anti-rat IgG antibody triggered rapid aggregation of human platelets, similar to mouse platelets (Figure 1D). Of note, this platelet response was not dependent on FcγRIIa as blocking this with IV.3 antibody did not prevent JAQ1-cross-linking induced platelet aggregation, but only minimally delayed it (Figure 1D, and Suppl. Figure 1A). Pre-incubation of human washed platelets with 5, 10 or 20 µg/ml JAQ1 reduced and delayed aggregation in response to CRP, but in contrast to mouse platelets (Figure 3E, and Suppl. Figure 3A,C) this was not abrogated. Notably, the traces of JAQ1-treated samples showed a reduced platelet shape change, pointing to a JAQ1-dependent platelet pre-activation and indicating that residual observable aggregation is partly due to this effect (Figure 1E, and Suppl. Figure 1B-C). Intriguingly, the presence of JAQ1 rather promoted collagen- and convulxin- dependent aggregate formation, and this occurred independently of FcγRIIa (Figure 1E, and Suppl. Figure1B-C), indicating that JAQ1 modulates huGPVI towards a pre-active state.

Treatment with higher concentrations of JAQ1 slightly exacerbated CRP-inhibitory effect and convulxin-dependent increased aggregation but not with collagen (Figure 1E, and Suppl. Figure 1B-C). Finally, we tested JAQ1 effect on spreading of human platelets on a fibrinogen-coated surface in absence of additional agonists. Interestingly, pre-incubation with JAQ1 but not control IgG increased the percentage of fully spread platelets (phase 4) on the surface (Figure 1F-G). Collectively, these data indicate that the binding epitope of JAQ1 may functionally not be fully conserved between mouse and human GPVI.

► **Figure 1. Anti-mouse GPVI monoclonal antibody JAQ1 binds to human GPVI and modulates its function**. (**A**) Washed human or mouse blood was pre-incubated with JAQ1 IgG-FITC and mean fluorescence intensity (MFI) was measured by flow cytometry; where indicated, human blood was pre-incubated with 20 µg/ml IV.3. Irrelevant rat-IgG-FITC was used as control. (**B**) Human blood was pre-incubated with 20 µg/ml EMF1, EMF-2 or control IgG for 10 min and subsequently incubated with JAQ1-FITC for 10 min, irrelevant IgG-FITC was used as control. (**C**) Mouse (*WT*) and human platelet lysates were separated by 10% SDS-PAGE under non-reducing condition and blotted onto PVDF membrane. JAQ1-HRP was used to detect GPVI on the membrane. GAPDH served as loading control. (**D**) Murine or human washed platelets were pre-incubated with either JAQ1 or a control IgG and aggregometry was performed; crosslinking of bound antibody was induced by adding an anti-Rat IgG antibody and light transmission was recorded for 15 min. When indicated, human platelets were incubated with IV.3 prior to JAQ1 addition. (**E**) Human washed platelets were pre-incubated with either JAQ1 or a control IgG and aggregometry was performed; aggregation was induced using the indicated agonists and for 10 min. (**F-G**) Human washed platelets were pre-incubated with IV.3 plus JAQ1 IgG or a control IgG and let spread on a 100 µg/ml fibrinogen-coated surface for 45 min at 37°C. Experiments shown are representative of n = 4. Flow cytometry and spreading data are expressed as means ± SD, significance is expressed as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. indicated group (t-test).



A humanized GP6 mouse line to study the effect of JAQ1 on huGPVI

In order to study JAQ1 effects on huGPVI in absence of possible FcγRIIa interferences, we capitalized on a newly generated mouse line humanized for the *GP6* gene (*hGP6*$^{tg/tg}$). The mouse line generation is thoroughly described in the methods and Suppl. Figure 4. To ensure suitability of the newly generated mouse line for further experiments, we analyzed platelet count, size and expression levels of prominent

membrane glycoproteins and found no alteration compared to wild-type controls, except for selective expression of human or mouse GPVI (Suppl. Tables 1 and 2). As expected, we also did not find any difference in overall platelet activation (Suppl. Figure 4A-B) and GPVI-dependent platelet aggregation and thrombus formation under flow (Suppl. Figure 4C-F). Next, we confirmed by flow cytometry that JAQ1 binds to platelets of *hGP6*$^{tg/tg}$, *hGP6*$^{wt/tg}$ and wild-type mice in a comparable manner (Figure 2A). Furthermore, we tested whether EMF-1 or EMF-2 compete with JAQ1 for binding to *hGP6*$^{tg/tg}$ platelets. In line with the results obtained with human platelets, JAQ1-FITC binding was profoundly reduced upon pre-incubating platelets with EMF-1 (~76.4% reduction) but very marginally by EMF-2 (~16%) (Figure 2B). To exclude that the inability of JAQ1 to recognize human GPVI in a Western blot analysis was due to species-specific glycosylation, we probed platelet lysates from *WT*, *hGP6*$^{tg/tg}$ and *hGP6*$^{wt/tg}$ animals with JAQ1. As expected, signals were only obtained in samples from *WT* or h*GP6*$^{wt/tg}$ mice, but not from h*GP6*$^{tg/tg}$ platelet lysates. Of note, less mGPVI was detected in h*GP6*$^{wt/tg}$ platelet lysates as compared to *WT* platelets (70% reduction). Likewise, EMF-1 recognized huGPVI in lysates of *hGP6*$^{tg/tg}$ as well as *hGP6*$^{wt/tg}$ platelets (Figure 2C). As expected, the signal obtained on *hGP6*$^{wt/tg}$ lysates was reduced (-49.3%) as compared to that of *hGP6*$^{tg/tg}$ platelet lysates (Figure 2D-E). Finally, pre-incubation with JAQ1 IgG did not induce evident platelet aggregation, whereas cross-linking of JAQ1 with a secondary anti-rat IgG antibody induced rapid aggregate formation of *hGP6*$^{tg/tg}$-derived platelets (Figure 2F, and Suppl. Figure 2). Overall, these data confirm specific JAQ1 binding to huGPVI and illustrate that the newly generated mouse line is a suitable model for testing huGPVI-targeting molecules.

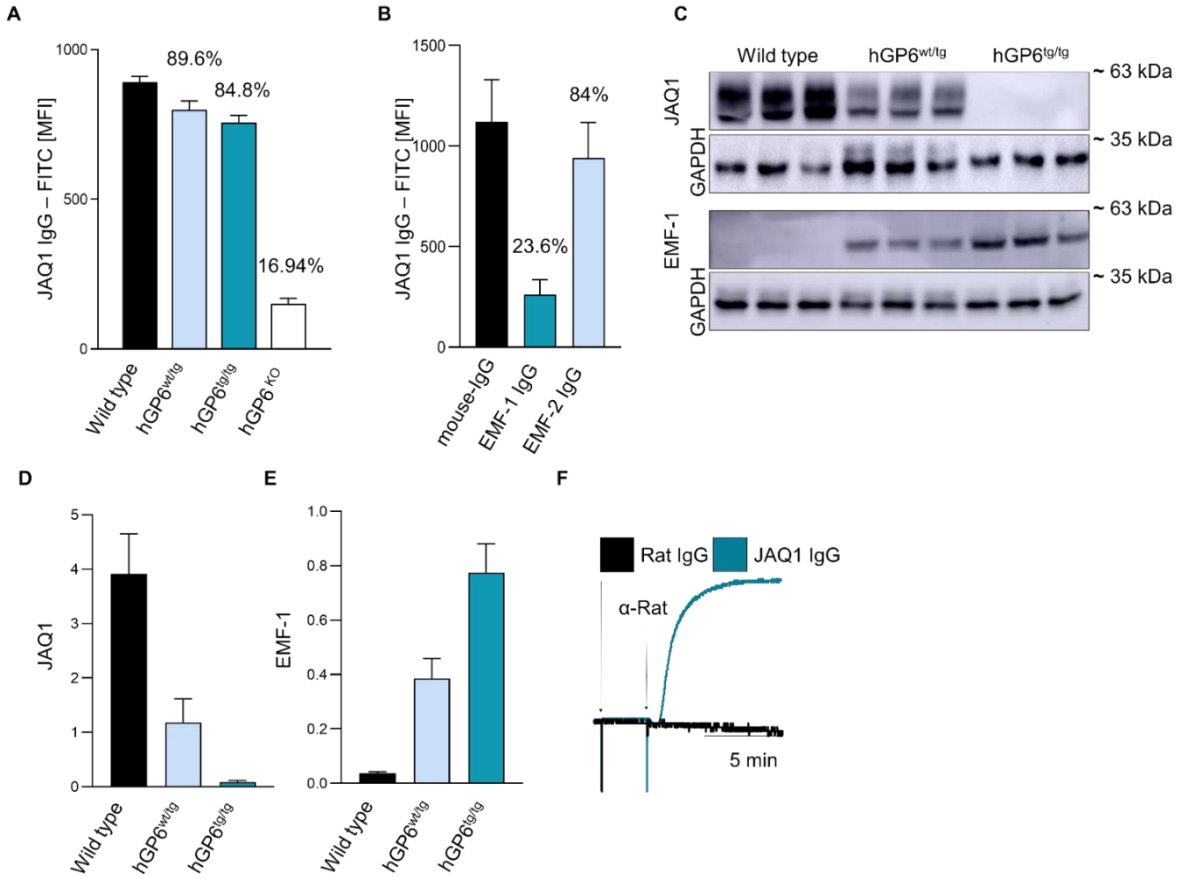


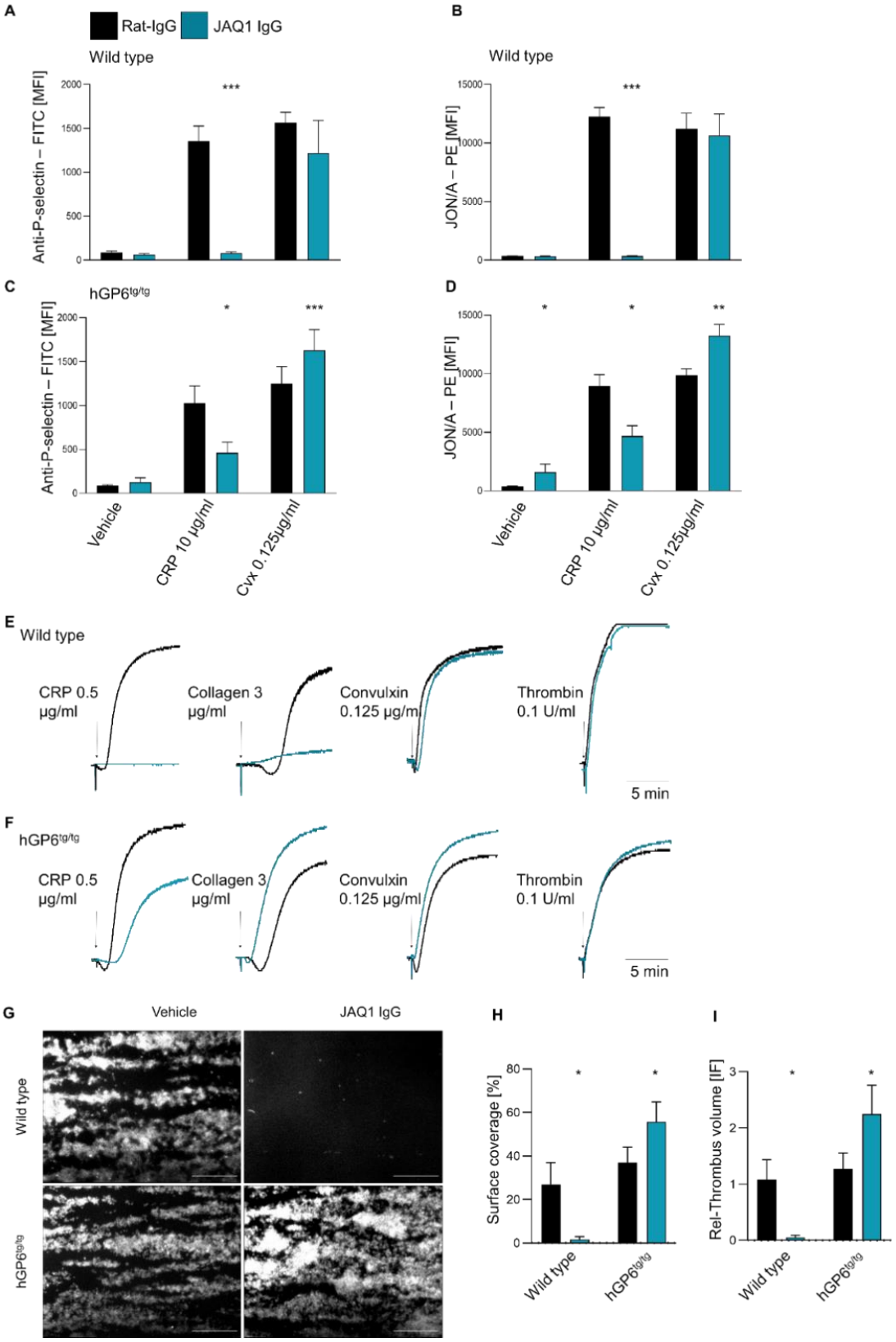
Figure 2. Use of *hGP6$^{tg/tg}$* mice confirms that JAQ1 binds to native human GPVI on the platelet surface, but not in Western blot analysis. (**A**) *WT*, *hGP6$^{wt/tg}$*, *hGP6$^{tg/tg}$* and *Gp6$^{-/-}$* washed blood was incubated JAQ1-FITC and MFI was measured by flow cytometry. (**B**) *hGP6$^{tg/tg}$* washed blood was pre-incubated with either EMF-1, EMF-2 or control IgG and subsequently incubated with JAQ1-FITC. (**C**) *WT*, *hGP6$^{wt/tg}$* and *hGP6$^{tg/tg}$* platelet lysates were separated by SDS-PAGE under non-reducing conditions and blotted onto a PVDF membrane. JAQ1 or EMF-1 were used to detect GPVI on the membrane. GAPDH served as loading control. (**D-E**) Western blot quantitative analysis relative to loading control. (**F**) *hGP6$^{tg/tg}$* washed platelets were pre-incubated with JAQ1 or control IgG and aggregate formation was induced using anti-rat IgG antibodies (20 µg/ml). Experiments shown are representative of n = 4, Western blot of n = 3. Flow cytometry and Western blot data are expressed as means ± SD.

Differential effect of JAQ1 on huGPVI and mGPVI

The effect of JAQ1 on GPVI-mediated platelet activation was assessed by flow cytometry using *WT* and *hGP6$^{tg/tg}$* platelets. In line with previous reports,[6] JAQ1

abrogated CRP-induced platelet activation of *WT* platelets, while convulxin (CVX)-induced activation remained intact (Figure 3A-B). In contrast, *hGP6$^{tg/tg}$* platelets displayed only moderately inhibited CRP-dependent platelet activation, while convulxin-induced integrin activation was even enhanced (Figure 3C-D). Next, the effect of JAQ1 on platelet aggregation was assessed. On *WT* platelets, JAQ1 abrogated CRP-induced and dramatically reduced collagen-induced aggregation, while aggregation to convulxin or non-GPVI agonist was unaltered (Figure 3E, Suppl. Figure 3A, C and data not shown). Interestingly, the aggregation of *hGP6$^{tg/tg}$* platelets in response to CRP was delayed and reduced following JAQ1-pretreatment. In stark contrast, JAQ1 accelerated and fostered collagen- and convulxin-induced *hGP6$^{tg/tg}$* platelet aggregation (Figure 3F, Suppl. Figure 3B, D), confirming our results with human platelets (Figure 1). To assess whether enhancement of platelet activation and aggregation would result in accelerated thrombus formation, we tested the effect of JAQ1 on *WT* and *hGP6$^{tg/tg}$* platelets in a whole mouse blood flow adhesion assay on a collagen-coated surface. As expected, JAQ1 completely abolished thrombus formation in *WT* blood. Strikingly however, the formation of thrombi was potently

► **Figure 3. Differential effect of JAQ1 on huGPVI and mGPVI**. (**A-D**) WT (**A, B**) or h*GP6$^{tg/tg}$* (**C, D**) diluted heparinized blood was pre-incubated with 20 µg/ml JAQ1 or control-IgG. Treated platelets were incubated with WUG 1.9-FITC (**A, C**), JON/A-PE (**B, D**) and stimulated with CRP (10 µg/ml), convulxin (1.25 µg/ml) or vehicle. (**E, F**) Washed platelets from WT (**E**) or h*GP6$^{tg/tg}$* (**F**) were pre-incubated with 20 µg/ml JAQ1 or control IgG and aggregation was induced with the indicated agonists; aggregation was measured for 10 min. (**G-I**) Heparinized WT or h*GP6$^{tg/tg}$* blood was pre-incubated with 20 µg/ml JAQ1 or control IgG and tested in flow adhesion assay on a collagen-coated surface. Percentage of the covered surface (H) and relative volume of thrombi (**I**) was analyzed based on fluorescence intensity of anti-GPIX-AF488 derivative. Experiments shown are representative of n = 4. Flow cytometry data are expressed as means ± SD, significance is expressed as * p <0.05, ** p <0.01, *** p <0.001 vs. indicated group (t-test).


A
Rat-IgG
JAQ1 IgG
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B
Wild type
JON/A – PE [MFI]
15000
10000
5000
0
***
C
hGP6tg/tg
Anti-P-selectin – FITC [MFI]
2000
1500
1000
500
0
*
***
Vehicle
CRP 10 µg/ml
Cvx 0.125µg/ml
D
JON/A – PE [MFI]
15000
10000
5000
0
*
*
**
Vehicle
CRP 10 µg/ml
Cvx 0.125µg/ml
E
Wild type
CRP 0.5 µg/ml
Collagen 3 µg/ml
Convulxin 0.125 µg/ml
Thrombin 0.1 U/ml
5 min
F
hGP6tg/tg
CRP 0.5 µg/ml
Collagen 3 µg/ml
Convulxin 0.125 µg/ml
Thrombin 0.1 U/ml
5 min
G
Vehicle
JAQ1 IgG
Wild type
hGP6tg/tg
H
Surface coverage [%]
80
60
40
20
0
*
*
Wild type
hGP6tg/tg
I
Rel-Thrombus volume [IF]
3
2
1
0
*
*
Wild type
hGP6tg/tg

enhanced after incubation of *hGP6*$^{tg/tg}$-derived blood, thus illustrating differential effects of JAQ1 on mouse versus human GPVI (Figure 3G-I).

Discussion

The monoclonal antibody JAQ1 has become a widely used tool for platelet research and its inhibitory effect on mouse GPVI has been thoroughly characterized. Here, we report for the first time, that this antibody also cross-reacts with huGPVI. Interestingly, however, our data suggests that the binding epitope is functionally different between mGPVI and huGPVI. While JAQ1 inhibits CRP-dependent platelet activation in both species (albeit at different levels), it differs in its effects on convulxin- and collagen-induced platelet activation (Figure 1-3). On murine platelets, JAQ1 blocks activation in response to low and intermediate collagen concentrations (Figure 3),[38] while it enhances collagen induced activation of huGPVI expressing platelets (Figure 1, 3, Suppl. Figure 1, 3). One explanation could be that JAQ1 stabilizes GPVI dimers (or clusters), which 'prime' GPVI for subsequent platelet activation, thereby accelerating and enhancing platelet activation. Of note, JAQ1 even triggers a pre-activation of human but not mouse GPVI resulting in subtle integrin activation (already in the absence of further agonists) as revealed by flow cytometry (Figure 3A-D).

Unexpectedly, our data show that JAQ1 binds to a conserved epitope in mouse and human GPVI that somehow evolved to differ in its functional significance. Mouse, rat and guinea pig diverged from human ~90 million years ago,[39] and are more closely related within each other with respect to other species such as dog and swine.[40] These data are in agreement with our flow cytometry results, showing no binding to GPVI on the platelet surface in these animals. Previous studies revealed discrepancies between mouse and human GPVI affinity to specific ligand and discussed possible differences with regard to the relevance of this receptor in thrombosis,[41] thus, underscoring the

need to utilize humanized animal models. Indeed, our newly generated mouse line allowed us to faithfully reproduce the results obtained with human platelets, thereby excluding possible overlapping effects of FcγRIIa, which is present in human but not mouse platelets and signals through a similar pathway as GPVI. This data clearly shows that the here reported *hGP6*$^{tg/tg}$ mice are a suitable model system for testing GPVI modulators *in vitro* and *in vivo*.

In conclusion, we present a previously undescribed and unexpected pre-activating effect of JAQ1 on huGPVI. This study also illustrated how the same epitope on human and mouse GPVI has genetically diverged during evolution leading to a different functional significance.

Materials and Methods

Antibodies and reagents

Collagen-related peptide (CRP) was a generous gift from Paul Bray (Baylor College, USA); Horm collagen was purchased from Takeda (Linz, Austria); convulxin, was purchased from Enzo Life Sciences (New York, NY, USA); thrombin was purchased from Roche Diagnostic (Mannheim, Germany); rabbit anti-GAPDH and rat anti-mouse IgG-HRP antibodies were purchased from Sigma-Aldrich (Steinheim, Germany); goat anti-rat IgG-HRP was purchased from Dianova (Hamburg, Germany); anti-rabbit IgG-HRP was purchased from Jackson Immuno (Suffolk, UK). For human blood collection, the S-monovettes 3.2% citrate and safety-fly-needles 21G were purchased by Sarstedt (Nümbrecht, Germany). Micro-cuvettes for aggregometry were purchased from Labitec (Ahrensburg, Germany). 5 ml polystyrene round-bottom tubes for flow cytometry were purchased from Corning incorporated (Ney York, NY, USA). Heparin was purchased from Ratiopharm (Ulm, Germany). JAQ1,[24] EMF-1,[30] EMF-2, JON/A,[42,43] and WUG

1.9, were generated in house. IV.3 antibody was purchased from Biozol (Eching, Germany).

Blood bonors and blood collection

Blood was obtained from healthy volunteers not undergoing anticoagulant or antiplatelet drugs for at least 4 weeks before recruitment. For the present study, the volunteers signed a written informed consent after approval by the Institutional Review Boards of the University of Würzburg and in accordance with the Declaration of Helsinki. Relevant guidelines and regulations were followed during for performing all described methods. Butterfly needles were used for the collection of blood by venipuncture; samples were collected into 9 ml tubes containing 3.2% trisodium citrate. For all studies, the blood was used withing 4 h from withdrawal and kept at room temperature.

Animals

Animals used in this study were matched based on genetic background, sex and age. Experiments with animals shown in this article had been previously approved by the district government of Lower Franconia (Regierung von Unterfranken) and performed following the current Animal Research: Reporting of In Vivo Experiments guidelines (https://www.nc3rs.org.uk/arrive- guide lines). In order to generate the humanized *GP6* (*hGP6*$^{tg/tg}$) mouse line, the cDNA expressing human GPVI (huGPVI) was inserted at level of the murine ATG of the mouse *Gp6* gene via CRISPR-Cas9 technology. The mutagenesis was carried out on the base of previous publications[44] via inserting the cDNA expressing huGPVI within the exon 1 of the *WT* gene, thus allowing selective expression of human but not mouse GPVI (Suppl. Figure 5). Mice were genotyped by PCR with the forward primer 5´- TGGCAAGAAGAGATAAGTGGTGGCT -3´, the

reverse primers 5´-CAGGTCACCTTCAGGACTCACCAAT-3´ for wild-type amplification and 5´- CAGACTTCTCTTCATGGCCGGGAT -3´ for transgenic mouse amplification. For experiments, venous blood was collected in 300 µl of 20 U/ml heparin via retro-orbital bleeding.

Measurement of platelet count and size

For assessment of platelet size and count, mice were bled into tubes coated with EDTA, parameters were measured at using an automated cell counter (ScilVet, Scil Animal Care Company, Germany).

Washed human and murine platelets

Human washed platelets were obtained as follows: 2 ml of ACD pH4.5 were added to the citrated blood and centrifuged for 20 min at 300 g and room temperature. Platelet-rich-plasma was collected in new 15 ml tubes and supplemented with 1/10 ACD, 0.02 U/ml apyrase (A6410, Sigma-Aldrich) and 0.1 µg/ml PGI_2 (P6188, Sigma-Aldrich). Platelets were pelleted by centrifugation for 10 min at 500 g, washed twice with Tyrode's buffer containing 0.02 U/ml apyrase/ml and 0.1 µg/ml PGI_2. Murine platelets were washed as previously described.[45]

Flow cytometry assays

Human blood diluted 1:10 and murine blood diluted 1:20 in Tyrodes' buffer without $CaCl_2$ was used for flow cytometry analysis. For platelet activation analysis, murine blood was diluted in Tyrodes' buffer + $CaCl_2$. JON/A-PE (Emfret Analytics, Eibelstadt, Germany) was used to detect activated integrin αIIbβ3,[42] whilst P-selectin exposure was detected with a specific anti-mP-selectin FITC-conjugated antibody WUG 1.9.[42] Diluted murine blood was incubated with either CRP (10 µg/ml), convulxin (1.25 µg/ml), thrombin (0.1 U/ml) or vehicle control, together with JON/A-PE mAb and anti-P-

selectin-FITC mAb for 12 min and finally diluted into 500 µl PBS. Measurement of the MFI was performed on a FACSCelesta (BD Biosciences).

Western blotting

Human and murine platelets were lysed at a concentration of 5 x 10^8/ml in IP buffer containing a protease inhibitor cocktail. Platelet lysates were mixed with loading buffer and pre-heated to 95°C for 5 min before loading in a 10% polyacrylamide gel and the immunoblotting was performed as previously described.[46,47]

Aggregometry assay

Human and murine washed platelets were resuspended at a concentration of 2 x 10^8/ml in Tyrode's buffer without $CaCl_2$. Aggregometry was performed as previously described.[48] Human or murine platelets were activated using 0.5 µg/ml Collagen-related-peptide (CRP), convulxin (0.125 µg/ml) or collagen 3 µg/ml (mouse) and 10 µg/ml (human). Measurements were performed using and APACT 4 aggregometer from Labitec (Ahrensburg, Germany).

Spreading assay

Human platelets were washed and resuspended at a concentration of 10^8/ml in Tyrode's buffer without $CaCl_2$. Washed platelets were incubated with either JAQ1 IgG or control rat IgG for 1 min and then pipetted onto a 100 µg/ml fibrinogen-coated surface. Platelet were let spread for 45 min. Fixation, coating and visualization were performed as previously described.[49]

Author Contributions: S.N. performed the experiments, analyzed the data, and wrote the manuscript. Andreas Starke performed experiments. J.W.M.H and M.J.E.K. provided supervision, analyzed data and did proofreading of the manuscript. B.N. and

D.S. designed research, analyzed data, provided expert supervision and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: SN is supported by the European Union's Horizon 2020 research and innovation program under the Marie Sklodowska-Curie grant agreement No.766118; and is enrolled in a joint PhD program at the Universities of Maastricht (The Netherlands) and Würzburg (Germany).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: All data are included in the manuscript as figures, tables or supplement tables.

Conflicts of Interest: The authors declare no conflict of interest.

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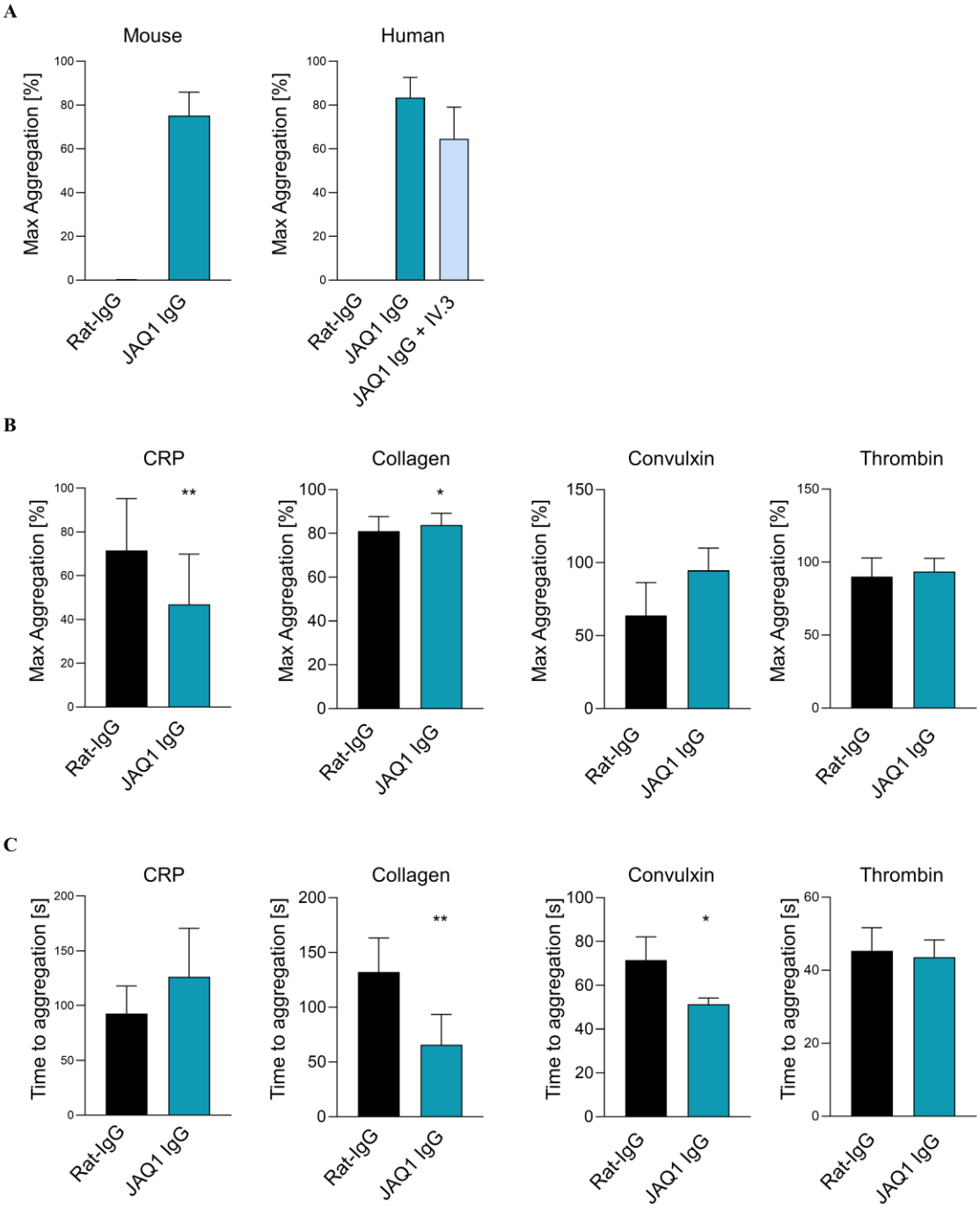
Supplemental Materials to Chapter 2

Suppl. Table 1. Platelet count and size. EDTA-treated blood was measured with an automated cell counter (ScilVet). Experiments shown are representative of n = 5. Flow cytometry data are expressed as means ± SD.

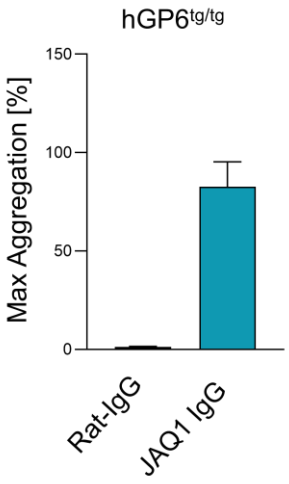
Parameter	Wild-type	h*GP6* $^{tg/tg}$	tTEST
Platelet count 10^3/mm^3	1084 ± 78	1212.75 ± 86.01	ns
Platelet Size (fl)	5.75 ± 0.21	5.8 ± 0.16	ns

Suppl. Table 2. Flow cytometric analysis of platelet glycoprotein expression. The expression levels of platelet glycoproteins were determined by flow cytometry. Experiments shown are representative for n = 5. Data are expressed as means ± SD, significance is expressed as * p <0.05, ** p <0.01, *** p <0.001 vs. indicated group (t-test).

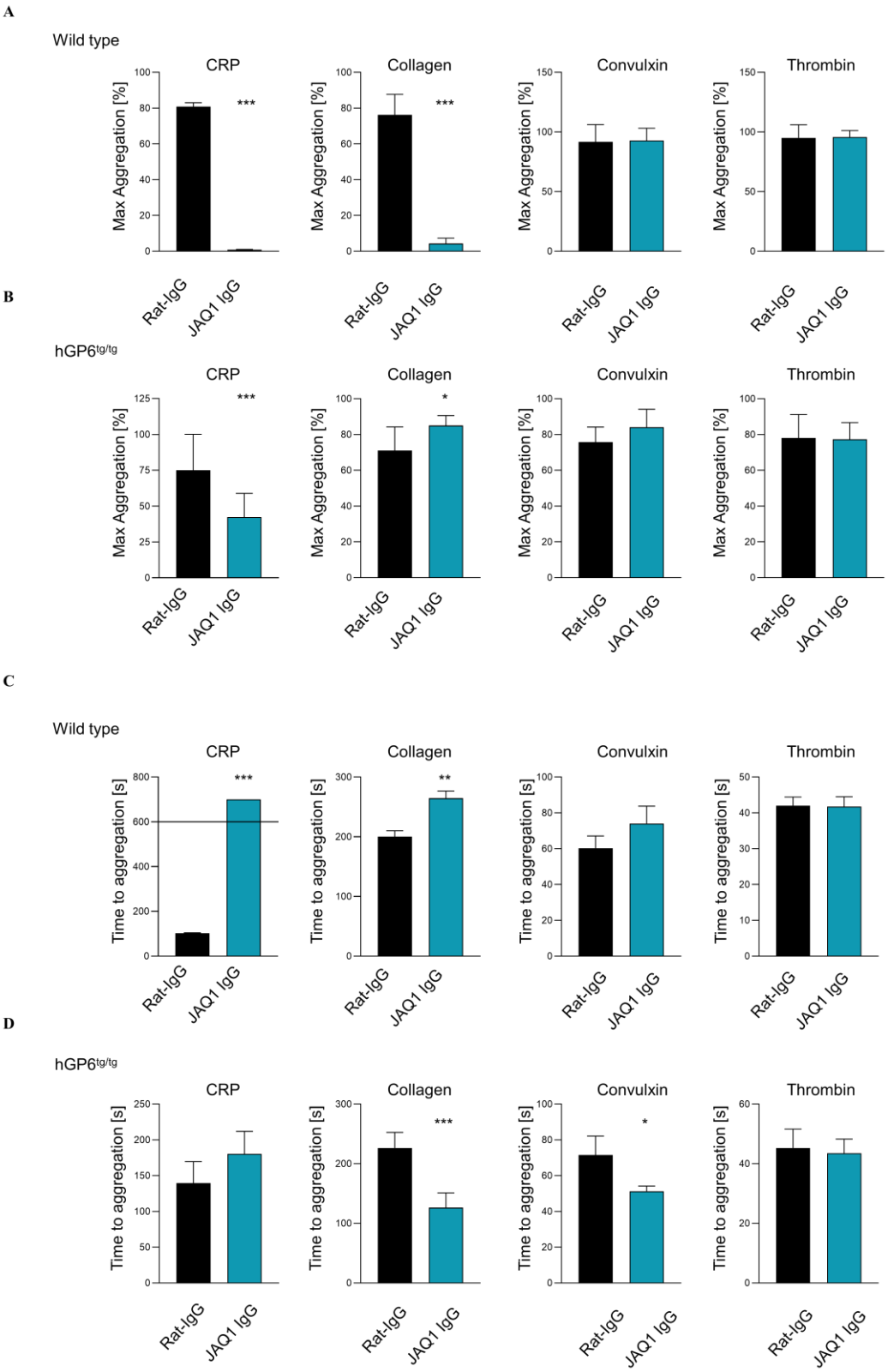
Glycoprotein	Wild-type (MFI)	h*GP6*$^{tg/tg}$ (MFI)	tTEST
GPIbβ	13072.5 ±523.7	13421 ± 909.1	ns
αIIbβ3	15043.5 ± 475.3	15740 ± 382.8	ns
α2β1	1570.25 ± 67.3	1530.25 ± 22.3	ns
α5β1	1038 ± 69.6	1031 ± 44.9	ns
GPIX	8257 ± 228.1	8336.75 ± 164	ns
β3	8590 ± 639.2	8065.75 ± 212.5	ns
GPV	8140.25 ± 184.5	8366 ± 117.3	ns
CD9	38258.5 ± 335.65	37954.5 ± 476.6	ns
mGPVI	790.5 ± 19.18	86.43 ± 19.19	***
huGPVI	223.5 ± 15.55	838.75 ± 32.81	***



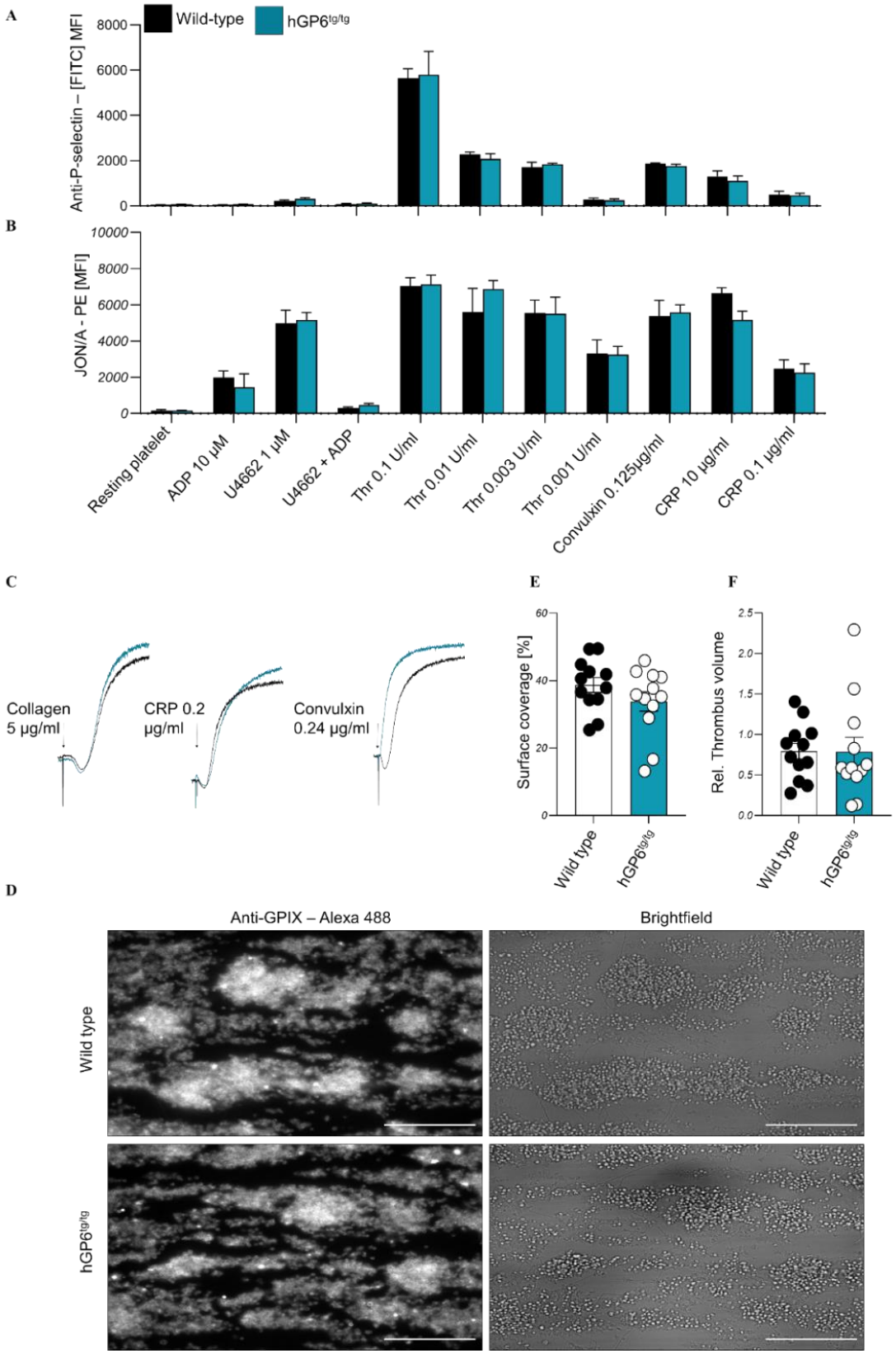
Suppl. Figure 1. Anti-mouse GPVI monoclonal antibody JAQ1 binds human GPVI and modulates receptor function. Analysis of light transmission aggregometry reported as maximal aggregation and time to aggregation (time to begin of aggregate formation). (**A**) Maximal aggregation relative to Figure 1D. (**B-C**) Analysis of aggregation curves shown in Figure 1E and reported as maximal aggregation (**B**) or time to start of aggregate formation (**C**). Experiments shown are representative of n = 4. Data are expressed as means ± SD.



Suppl. Figure 2. A humanized GP6 mouse line to study the effect of JAQ1 on huGPVI. Analysis of light transmission aggregometry shown in Figure 2F and reported as maximal aggregation. Experiments shown are representative of n = 4. Data are expressed as means ± SD.

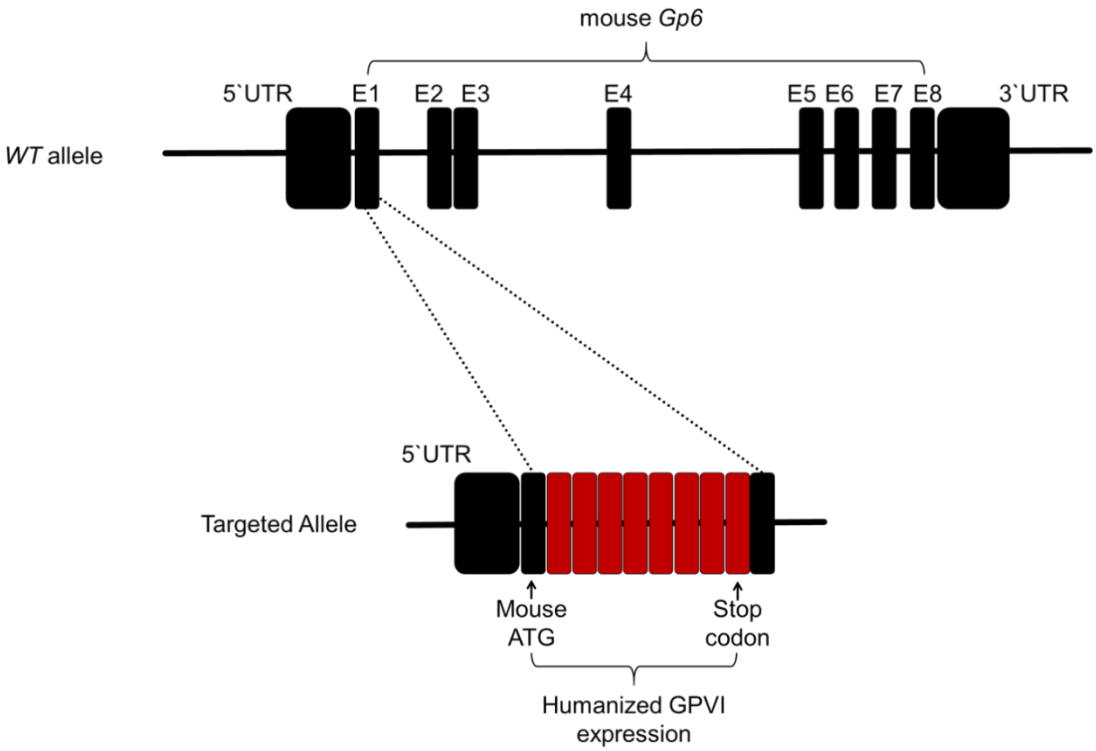


Suppl. Figure 3. Differential effect of JAQ1 on huGPVI and mGPVI. Analysis of light transmission aggregometry reported as maximal aggregation (**A-B**) and time to aggregation (time to begin of aggregate formation). (**C-D**). Experiments shown are representative of at n = 4. Data are expressed as means ± SD.



Suppl. Figure 4. Differential effect of JAQ1 on huGPVI and mGPVI. (**A-B**) Washed *WT* or *hGP6$^{tg/tg}$* blood was pre-incubated with the indicated agonists or vehicle. Platelet

◀degranulation (a) and integrin αIIbβ3 activation (b) were measured by flow cytometry. (**C**) Washed platelets from *WT* or *hGP6*$^{tg/tg}$ were tested in standard aggregometry using the indicated agonists; aggregation was measured for 10 min. (**D-F**) Heparinized *WT* or *hGP6*$^{tg/tg}$ blood was tested in a whole blood flow adhesion assay on a collagen-coated surface. Percentage of the covered surface (**E**) and relative volume of thrombi (**F**) was analyzed based on fluorescence intensity of anti-GPIX^{AF488} derivative. Experiments shown are representative of at least n = 4. Flow cytometry data are expressed as means ± SD.



Suppl. Figure 5. Generation of mutant mice. For the generation of humanized GPVI (*hGP6*$^{tg/tg}$) mice, the cDNA expressing human GPVI (huGPVI) shown in the figure in red, was inserted at the ATG site of the mouse *Gp6* gene (black) via CRISPR-Cas9 technology. Human *GP6* cDNA carries a stop codon thus allowing only selective expression of huGPVI.

Chapter 3

Temporal Roles of Platelet and Coagulation Pathways in Collagen- and Tissue Factor-Induced Thrombus Formation

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Int. J. Mol. Sci. 2022, 23(1), 358

I performed the experiments, analyzed the data, and wrote the manuscript. D.S. provided supervision. B.N. provided supervision and provided the anti-hGPVI antibody.J.W.M.H. and M.J.E.K. provided expert supervision for the flow-adhesion assay and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Abstract

In hemostasis and thrombosis, the complex process of thrombus formation involves different molecular pathways of platelet and coagulation activation. These pathways are considered as operating together at the same time, but this has not been investigated. The objective of our study was to elucidate the time-dependency of key pathways of thrombus and clot formation, initiated by collagen and tissue factor surfaces, where coagulation is triggered via the extrinsic route. Therefore, we adapted a microfluidics whole-blood assay with the Maastricht flow chamber to acutely block molecular pathways by pharmacological intervention at desired time points. Application of the technique revealed crucial roles of glycoprotein VI (GPVI)-induced platelet signaling via Syk kinase as well as factor VIIa-induced thrombin generation, which were confined to the first minutes of thrombus buildup. A novel anti-GPVI Fab EMF-1 was used for this purpose. In addition, platelet activation with the protease-activating receptors 1/4 (PAR1/4) and integrin αIIbβ3 appeared to be prolongedly active, and extended to later stages of thrombus and clot formation. This work thereby revealed a more persistent contribution of thrombin receptor- than of collagen receptor-induced platelet activation to the thrombotic process.

Keywords: coagulation, fibrin, glycoprotein VI, platelet receptors, spatiotemporal thrombus, thrombin

Introduction

In hemostasis, thrombosis, and thrombo-inflammation, multiple platelet and coagulation activation processes interact to establish the formation of a thrombus composed of aggregated and contracted platelets connected by a fibrin network or clot.[1-3] Consolidation of the thrombus is considered to be regulated by the release of paracrine platelet agonists and by local thrombin activity.[4,5] In the past years, several key molecular actors of the thrombus-forming process have been identified. The first interaction of platelets with exposed vascular collagen occurs via glycoprotein Ib-V-IX (GPIb)-dependent platelet adhesion to collagen-bound von Willebrand factor (VWF). The initial adhesion facilitates the interaction of glycoprotein VI (GPVI) with collagen. Mouse thrombosis models have confirmed the key role of platelet GPVI as a signaling collagen receptor, acting via protein tyrosine kinases such as Syk and culminating in the activation of phospholipase Cγ2 and phosphoinositide 3-kinases.[6-9] Recently, the key role of GPVI in thrombus formation was confirmed by studies with blood from patients with a congenital deficiency in the gene *GP6*].[10] Both in mouse and human, GPVI-induced signaling leads to platelet granule release, activation of integrin αIIbβ3, platelet shape change, and surface exposure of the procoagulant phospholipid phosphatidylserine.[11,12] For a stable platelet adhesion to collagen, in addition, binding via the integrins α2β1 and αIIbβ3 is required.[6] In thrombosis and hemostasis, the binding of fibrinogen to activated integrin αIIbβ3 on adjacent platelets generates a scaffold for the formation of platelet aggregates and, in flowing blood, for the buildup of a thrombus.[2,11,13]

Regarding the initiation of coagulation, vascular exposed tissue factor (TF) acts as an initial trigger of the extrinsic pathway and stimulates the proteolytic coagulation cascade of factor VIIa, factor Xa and thrombin.[14-17] The exposed phosphatidylserine on highly activated platelets enhances the generation thrombin to produce sufficient amounts for the formation

of a fibrin clot.[12,18-20] It is stipulated,[21] but incompletely studied that the generated thrombin stimulates human platelets by targeting the PAR1 and PAR4 receptors. Additional platelet activation is achieved by platelet-derived autocoids, which enhance the activation of αIIbβ3 and thus mediate further thrombus growth.[22,23] The thrombin-dependent formation of fibrin fibers consolidates the thrombus, converting it into a vessel-occlusive clot.

Using microfluidic approaches, we and others have shown that immobilized TF in a dose-dependent way enhances the platelet activation in thrombus formation and triggers the formation of a fibrin clot.[24-26] To investigate the molecular pathway in that process, we developed and validated a microfluidics test, operating in combination with multicolor microscopy, which provided multiparameter information on the formation of platelet-fibrin thrombi during whole-blood perfusion.[26] These microfluidic studies showed that collagen-induced GPVI signaling led to thrombi with patches of phosphatidylserine-exposing platelets, which acted as a driving force for ensuing thrombin and fibrin generation.[27,28] Recent studies also revealed an intricate molecular synergy between the actions of immobilized collagen and TF, in that TF alone was unable to support platelet adhesion, while collagen alone was a poor supporter of the clot-forming process.[26] However, the precise time frame by which TF and collagen, and by consequence the molecular actions of PARs and GPVI operate, has remained unclear. Knowledge of this is becoming important with the new finding that GPVI also mediates platelet adhesion and activation to fibrin.[29-31]

In recent years, many studies have sought to unravel the molecular and biochemical mechanisms leading to an optimal hemostatic response after vascular damage and to provide insights into the spatiotemporal regulation of platelet activation, aggregation, coagulation, and thrombus formation using, for instance, *in vivo* imaging [32] and mathematical modeling.[33] It is then silently assumed that the various platelet receptors and their downstream signaling pathways are in continuous operation during the whole period

of thrombus buildup and fibrin clotting. However, if this is really the case is yet to be elucidated.

For the present paper, we hypothesized that the molecular pathways of flow-dependent platelet activation during the formation of a thrombus and clot are time-restricted. On the basis of prior experiments, we considered that the first two min of flow are critical for an initial stage of thrombus formation.[26] To investigate the time-dependent role of these pathways in more detail, we developed a procedure using the Maastricht flow chamber, in which during continuous blood flow specific inhibitors could be introduced acutely at a requested time point, typically after 2 min of start. To do so, we modified a previously standardized microfluidic device containing collagen and collagen/TF microspots.[34] By real-time microscopic examination of platelet adhesion, platelet aggregation, and fibrin formation under high-shear flow, we then sought to distinguish between early (<2 min) and later (2–10 min) contributions of the examined pathways relevant for thrombus formation at arterial flow conditions.

Results

Time-restricted roles of tissue factor and factor VIIa in collagen-dependent formation of platelet-fibrin thrombi

In order to distinguish between early and late contributions of the thrombus pathways of interest, we needed to adapt the earlier used microfluidic system. Changes entailed the insertion of a triple inlet tubing system, where each of the three inlet tubes was connected to a 1 ml plastic syringe, the first of which contained recalcification medium, the second untreated blood, and the third inhibitor-treated blood (see Methods). The flow perfusion rate with each syringe was controlled with a pulse-free nanopump. The tubing system and the precise flow chamber inlet dimensions allowed for an instant and complete mixing of (control or inhibited) blood with the recalcification medium. By using this three-way inlet

system, we thus could keep a continuous blood flow during the change from control to inhibited blood samples, for which change was by default achieved by switching between pumps 2 and 3 after 2 min of initial blood flow.

After checking of proper functioning of the triple inlet system, i.e., the complete blood mixing with recalcification medium (data not shown), we performed a series of experiments where blood samples were flowed over microspot pairs of collagen and collagen/TF at a final shear rate of 1000 s^{-1}. The two blood samples with or without inhibitor were equally pre-labeled with $DiOC_6$ to detect adhered platelets, with Alexa Fluor (AF)568 annexin A5 to identify phosphatidylserine-exposing platelets, and with AF647-fibrinogen to detect fibrin formation, as detailed previously.[26] Following this process, at 2 min intervals, brightfield and multicolor fluorescence microscopic images were taken from each microspot. The images were analyzed in a standard way to produce a total of seven platelet, thrombus, and coagulation parameters (P1-7) per time point and per surface type (Table 1).

Parameter	Time (min)	Image type	Description	Unit
Platelet parameter				
P1	2-10	$DiOC_6$	platelet adhesion	% SAC
Thrombus parameters				
P2	2-10	brightfield	thrombus coverage	% SAC
P3	2-10	brightfield	thrombus morphology	score 0-5
P4	2-10	brightfield	thrombus contraction	score 0-3
P5	2-10	brightfield	thrombus multilayering	score 0-3
Coagulation parameters				
P6	2-10	AF568-annexin A5	PS exposure	% SAC
P7	2-10	AF647-fibrin(ogen)	fibrin deposition	% SAC

◄ **Table 1.** Parameters and microscopic image sources of thrombus formation. Seven parameters were obtained per whole-blood flow run at time points 2, 4, 6, 8, and 10 min. These were categorized into platelet (P1), thrombus (P2–5), and coagulation-related parameters (P6–7). Abbreviations: PS, phosphatidylserine; SAC, surface area coverage.

As usual, the perfusion of control (vehicle) blood over collagen/TF microspots resulted in fast platelet adhesion, followed by platelet aggregation and formation of contracting thrombi. The thrombi contained phosphatidylserine-exposing platelets, which at later time points were surrounded by fibrin fibers (Figure 1A). In the absence of TF, smaller and less contracted platelet aggregates were formed, while essentially no fibrin was seen during the first 10 min (Figure 1B). These observations were in agreement with previous findings.[26] Quantification of the images from the collagen/TF surface, taken over time, showed a consistent and gradual increase in thrombus coverage (P2) and in fibrin deposition (P7), starting at around 4 min (Figure 1C,D). In the absence of TF, the process of thrombus formation was slower in onset. Heatmap representation of the univariate scaled parameters P1-7 up to 10 min illustrated a decelerating effect on collagen-only microspots for essentially all parameters, except for phosphatidylserine exposure (Figure 1D). This was also concluded from a subtraction heatmap of the scaled parameters vs. those of collagen/TF microspots (vehicle condition) (Figure 1E). Together, this analysis pointed to an overall stimulating role of TF on platelet deposition, activation, and aggregation, as well as on thrombus consolidation and fibrin clotting.

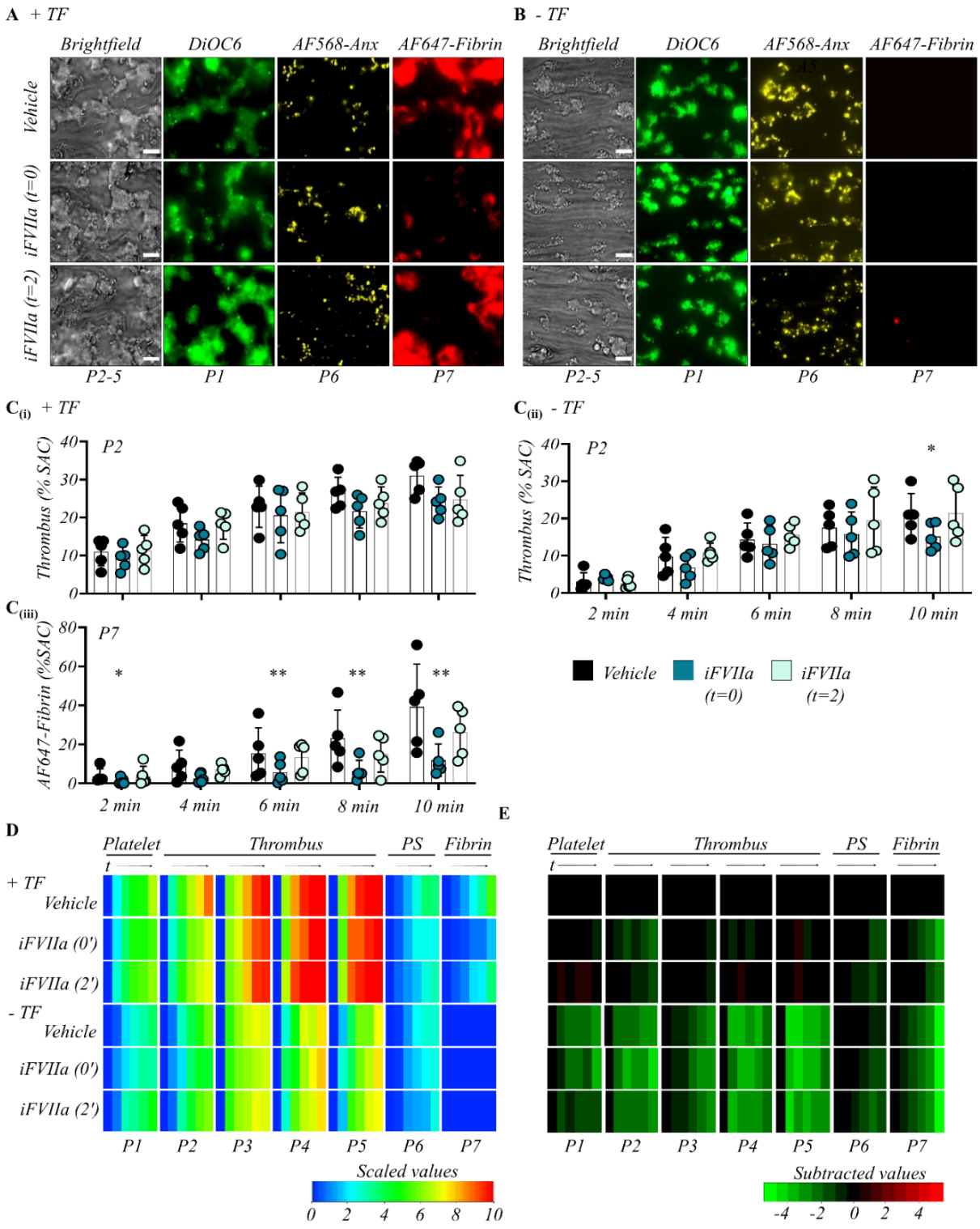


Figure 1. Early contributions of TF and factor VIIa in whole-blood thrombus formation. Citrated whole blood samples from healthy subjects (n = 5) were supplemented with fluorescent labels to simultaneously detect platelet adhesion (P1, $DiOC_6$), thrombus and platelet multilayer characteristics (P2–5, brightfield), phosphatidylserine exposure (P6, AF568-annexin A5), and fibrin deposition (P7, AF647-fibrin). Using a three-way tubing inlet system, allowing complete

◄fluid mixing, we co-infused blood samples with recalcification medium and perfused them through a parallel-plate flow chamber containing microspots of collagen (upstream) and collagen/TF (downstream) at a wall-shear rate of 1000 s^{-1}. During blood flow, monochromatic images in 4 colors were captured by brightfield and fluorescence microscopy at 2, 4, 6, 8 and 10 min. Where indicated, the perfusion was using iFVIIa-treated blood (1 µM, f.c.) from the start (t = 0 min), or the iFVIIa-treated blood was introduced after 2 min. Control runs were carried out with blood samples containing vehicle solution. (**A-B**) Representative 10-min end-stage images of vehicle control, early extrinsic pathway inhibition (iFVIIa from start), and later extrinsic pathway inhibition (iFVIIa from 2 min). Images were from collagen microspots in the presence (**A**) or absence (**B**) of TF. Bars = 20 µm. Quantitative analysis from collagen ± TF surfaces of parameter P2: thrombus coverage (**C i**, **ii**) and P7: fibrin deposition (**C iii**). Fibrin staining was essentially absent on collagen-only microspots. Means ± SD, *p <0.05, **p <0.01 vs. indicated group (*t*-test). (**D**) Heatmap of univariate scaled values per parameter P1–7 for indicated surfaces and conditions. (**E**) Subtraction heatmap of scaled parameters versus vehicle control (collagen/TF), filtered for relevant changes with p <0.05; color codes as indicated in color bars. For additional dataset, see Suppl. Figure 1.

It is well known that TF triggers the clotting by forming a complex with factor VII(a) and factor Xa, which mediates the generation of more factor Xa and thrombin.[11] For the microspots of collagen/TF, we investigated the time-dependency of this role of TF by treating the blood with inhibited factor VIIa (iFVIIa). In the experiments, iFVIIa was either present from the start, or was introduced after 2 min by pump switching. The chosen concentration of 1 µM iFVIIa has been shown to block all clotting-related TF activity under high-shear flow conditions.[26]

For collagen/TF microspots, the initial presence of iFVIIa insignificantly reduced thrombus coverage (P2) and other thrombus parameters, while it completely and significantly suppressed fibrin formation to a level resembling that of collagen-only surfaces (Figure 1A–D). The iFVIIa effects were clearly apparent from a subtraction heatmap presentation of all parameters P1-7 over time (Figure 1E). Markedly, the inclusion of iFVIIa after 2 min did no longer influence any of the thrombus and fibrin parameters (Figure 1D,E). In comparison, for the collagen-only surfaces, the early presence of iFVIIa (0 min) only had a significant reducing effect on thrombus coverage at the latest time point, while the iFVIIa

did not affect platelet adhesion and phosphatidylserine exposure (Suppl. Figure 1). Moreover, the late presence of iFVIIa was without effect here. Together, these collagen/TF data pointed to a time-restricted role of the TF-factor VIIa-driven coagulation pathway in thrombus consolidation and fibrin deposition. A residual effect of iFVIIa in the absence of coated TF has been noticed before and is explained by redundancy between the intrinsic and extrinsic pathways of thrombin generation.[26]

Longer-term roles of receptors PAR1 and PAR4 in formation of platelet-fibrin thrombi

Earlier data with collagen-only microspots pointed to a slow onset of thrombin generation via the intrinsic pathway.[26] Given the high responsiveness of platelets to thrombin acting via Gqα-protein-coupled receptors PAR1 and PAR4,[35] we then unraveled the time-dependency of this thrombin receptor pathway. For this purpose, we blocked both PAR1 and PAR4 using 2 μM atopaxar (f.c.) and 1 μM BMS-986120 (f.c.), respectively. Using whole-blood flow cytometry, we checked that these doses were optimal for suppressing thrombin-induced platelet activation (J. Zou, unpublished data, 2021).

For the collagen/TF surfaces, initial blockage of PAR1+4 resulted in substantial and significant decreases in platelet adhesion (P1), thrombus growth (P2), and fibrin formation (P7) (Figure 2A-E). In contrast, phosphatidylserine exposure (P6) was unaltered (Suppl. Figure 3A,C). Regarding thrombus contraction (P4) and multilayering (P5), the early PAR1+4 inhibition caused marked and significant reductions over time, approaching the thrombus parameters in the absence of TF (Suppl. Figure 3E, G).

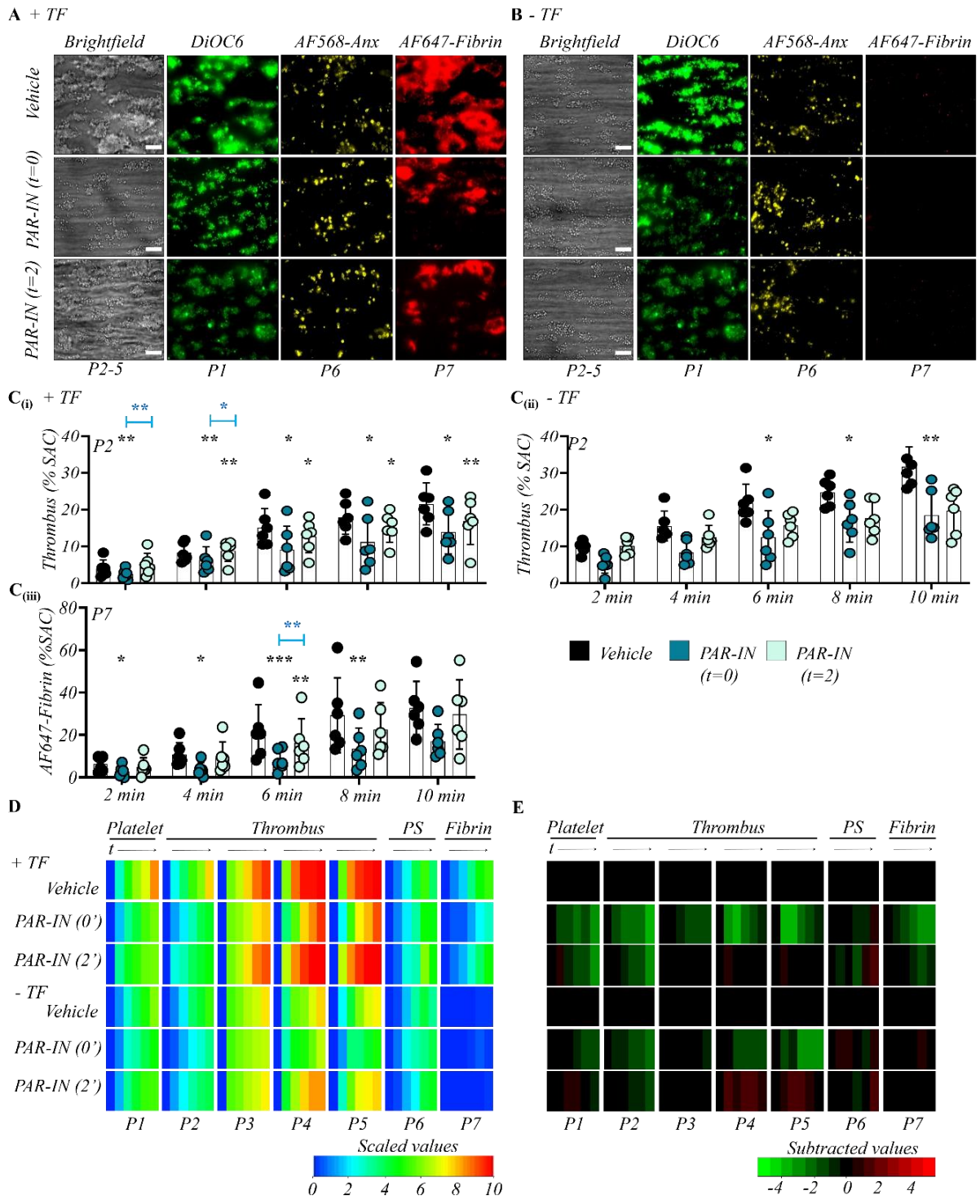


Figure 2. Involvement of platelet thrombin receptors PAR1 and PAR4 in thrombus formation. Citrated whole-blood samples from healthy subjects (n = 6) were supplemented with fluorescent labels and perfused over microspots of collagen and collagen/TF, as shown in Figure 1. Where indicated (PAR-IN), perfusion was switched from control blood to blood preincubated with vehicle or a mix of atopaxar (PAR1 inhibitor, 2 µM, f.c.) and BMS-986120 (PAR4 inhibitor, 1 µM, f.c.). Control blood runs were carried out with vehicle solution. Thrombus formation on both microspots was analyzed from captured images for parameters P1-7, as in Figure 1. (**A**, **B**) Representative 10

◄min end stage images of vehicle control condition; early PAR-IN (mix from start); later PAR-IN (mix from 2 min). Images were taken from microspots in the absence (**A**) or presence (**B**) of TF. Quantitative analysis from ± TF surfaces of parameter P2: thrombus coverage (**C i**, **C ii**), and P7: fibrin deposition (**C iii**). Means ± SD, *p <0.05, **p <0.01, ***p <0.001 vs. indicated group (*t*-test). (**D**) Heatmap of univariate scaled time-dependent values of P1–7 for indicated surfaces and conditions. (E) Subtracted heatmap of scaled parameters versus collagen and collagen/TF control runs, further as for Figure 1. For additional data, see Suppl. Figure 2.

A different pattern of changes was obtained when PAR1+4 were inhibited after 2 min. On collagen/FT, the later intervention continued to reduce the thrombus growth (Figure 2A,C), but the thrombus characteristics (P3–5) remained unaltered. Markedly, fibrin formation (P7) was still moderately reduced at 6–8 min, which was linked to the reduction in thrombus size (Figure 2C).

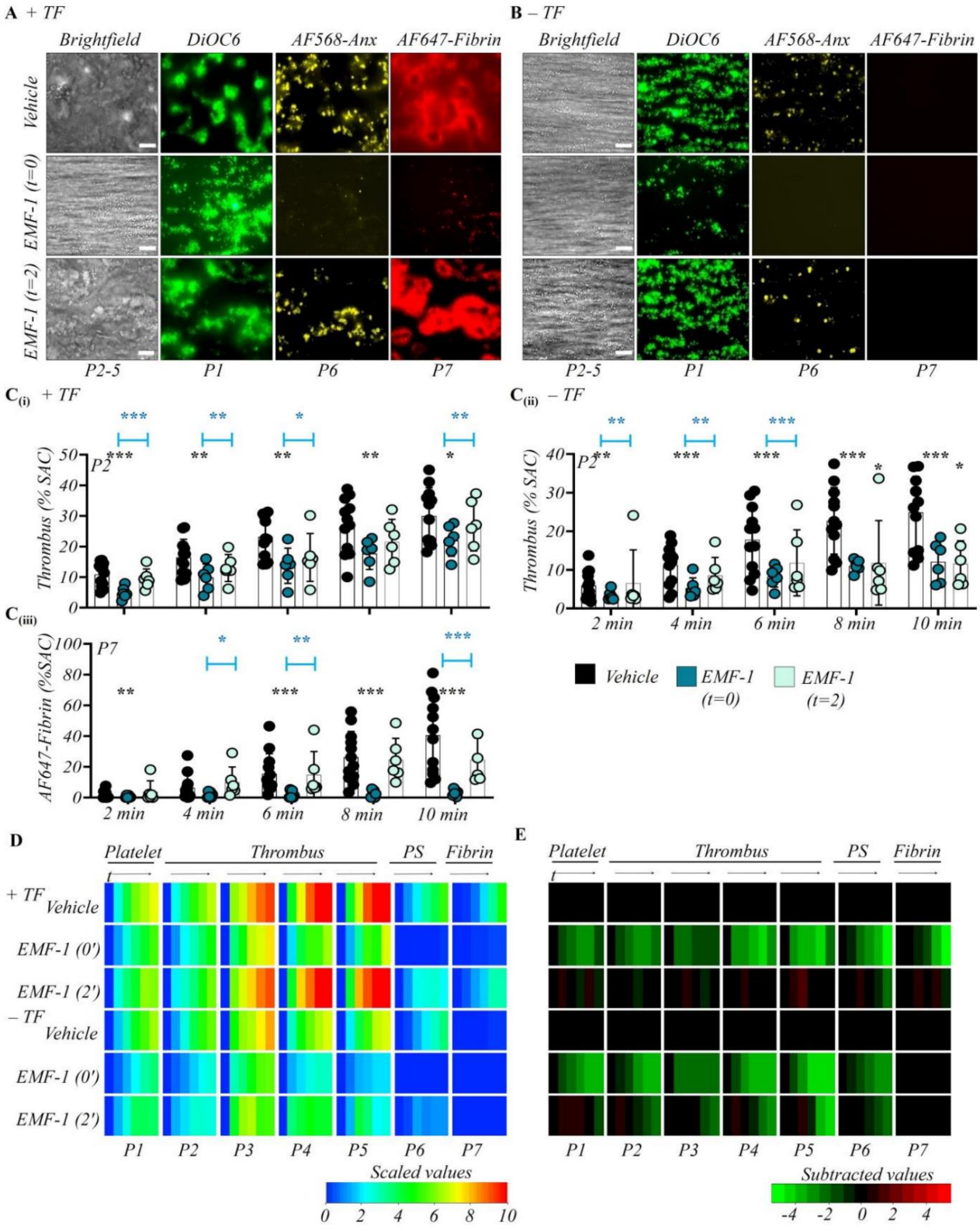
Regarding collagen-only microspots, initial inhibition of the PAR1+4 caused a reduction of platelet adhesion (P1), thrombus coverage (P2), and other thrombus characteristics (P4–5), which was seen at all time points but was not always significant (Figure 2B–E). No such effects were seen upon late PAR1+4 inhibition (Figure 2E).

Similarly, no effect was detected on platelet phosphatidylserine exposure (Suppl. Figure 2). Interestingly, these effects of PAR1+4 inhibition mostly phenocopied the effects of absence of TF. Jointly, the results indicated a role of PAR1+4 on initial thrombus progression and contraction, especially in the presence of TF, and hence pointed to a most pivotal role of thrombin-induced signaling in the early phase of thrombus formation, but with a residual effect at later stages of the process. This implies a continued co-operation between coagulation pathways and platelet activation processes during thrombus growth.

Initial contribution of GPVI and downstream tyrosine kinase signaling in formation of platelet-fibrin thrombi

We subsequently investigated the early and later roles of collagen receptor GPVI in thrombus formation using the same intervention setup. To functionally block GPVI, we used a novel, recently characterized anti-human GPVI Fab fragment EMF-1 at optimal concentration of 10 µg/ml (Emfret Analytic, Eibelstadt/Würzburg, Germany, unpublished data, B. Nieswandt, June 2021). For collagen/TF surfaces, the initial GPVI blockage led to a formation of smaller and unstable thrombi when compared to the vehicle control condition (Figure 3A). A similar change was observed for collagen-only surfaces (Figure 3B). On either surface type, platelet adhesion (P1) was partly reduced, while phosphatidylserine exposure (P6) was almost completely annulled (Suppl. Figure 3A-D). These findings agreed with the understanding that exposure of phosphatidylserine is a marker of collagen-induced GPVI signaling.[36] Quantitative image analysis revealed that the blockage of GPVI with EMF-1 Fab from start led to a robust reduction of the majority of platelet activation and thrombus parameters (P2-6) on collagen ± TF surfaces (Figure 3C-E). In the presence of TF, fibrin deposition (P7) was abolished as well (Figure 3), which agreed with the procoagulant effect of phosphatidylserine exposure in the flow assay.[26]

►**Figure 3. Time-dependent contribution GPVI in thrombus formation.** Citrated whole blood was labeled and co-perfused with recalcification medium over collagen and collagen/TF microspots (n = 6). Where indicated, perfusion was switched from control blood to blood preincubated with vehicle or anti-GPVI Fab EMF-1 (10 µg/ml, f.c.). Thrombus parameter analysis and heatmap presentation were as for Figure 1. (**A**, **B**) Representative end stage images of vehicle control condition; early GPVI inhibition (EMF-1 Fab from start); later GPVI inhibition (EMF-1 Fab from 2 min). Images from microspots without (**A**) or with (**B**) TF; bars = 20 µm. Quantitative analysis from ± TF surfaces of parameter P2: thrombus coverage (**Ci**, **C ii**), and P7: fibrin deposition (**C iii**). Means ± SD, *p <0.05, **p <0.01, ***p <0.001 vs. indicated group (*t*-test). (**D**) Heatmap of univariate scaled time-dependent values, and (**E**) subtracted heatmap of scaled parameters versus control runs, as for Figure 1. For additional data, see Suppl. Figure 3.


A + TF
B – TF
Brightfield
DiOC6
AF568-Anx
AF647-Fibrin
Vehicle
EMF-1 (t=0)
EMF-1 (t=2)
P2-5
P1
P6
P7
C(i) + TF
C(ii) – TF
C(iii)
Thrombus (% SAC)
AF647-Fibrin (%SAC)
2 min
4 min
6 min
8 min
10 min
Vehicle
EMF-1 (t=0)
EMF-1 (t=2)
D
E
Platelet
Thrombus
PS
Fibrin
+ TF Vehicle
EMF-1 (0')
EMF-1 (2')
– TF Vehicle
EMF-1 (0')
EMF-1 (2')
Scaled values
Subtracted values

We then determined how GPVI blockage with EMF-1 Fab influenced the process, when introduced after 2 min of perfusion. In the absence of TF, this late intervention led to a moderate reduction in ensued thrombus formation. With TF present, fibrin clotting was unaffected (Figure 3C). Heatmap-based analysis showed that the thrombus parameters (P2–5) were moderately reduced in the absence of TF (Figure 3D-E). To a certain extent, the reduction in phosphatidylserine exposure also persisted (Suppl. Figure 3C-D). Together, this pointed to a mostly early pivotal role of GPVI-induced platelet activation in thrombus formation and clotting. At later phase, residual GPVI-mediated effects could apparently be taken over by TF, and hence thrombin generation.

We also investigated the role of the protein tyrosine kinase Syk as a signaling regulator directly downstream of GPVI. Therefore, we used the inhibitor PRT-060318 (Syk-IN, 20 μM) at a concentration previously shown to completely abrogate the collagen- and GPVI-induced signaling under flow.[37] Examination of end-stage microscopic images showed that the early intervention with Syk-IN caused similar reductions in thrombus size, phosphatidylserine exposure, and fibrin formation (Figure 4A-B), as were observed with anti-GPVI EMF-1 Fab. On either surface (collagen/TF or collagen-only), Syk-IN slightly affected platelet deposition, but it completely abolished the phosphatidylserine exposure and platelet aggregation (Suppl. Figure 4A, D). Furthermore, in the presence of TF, Syk-IN also annulled fibrin deposition, while all thrombus parameters were strongly downregulated (Figure 4C-E).

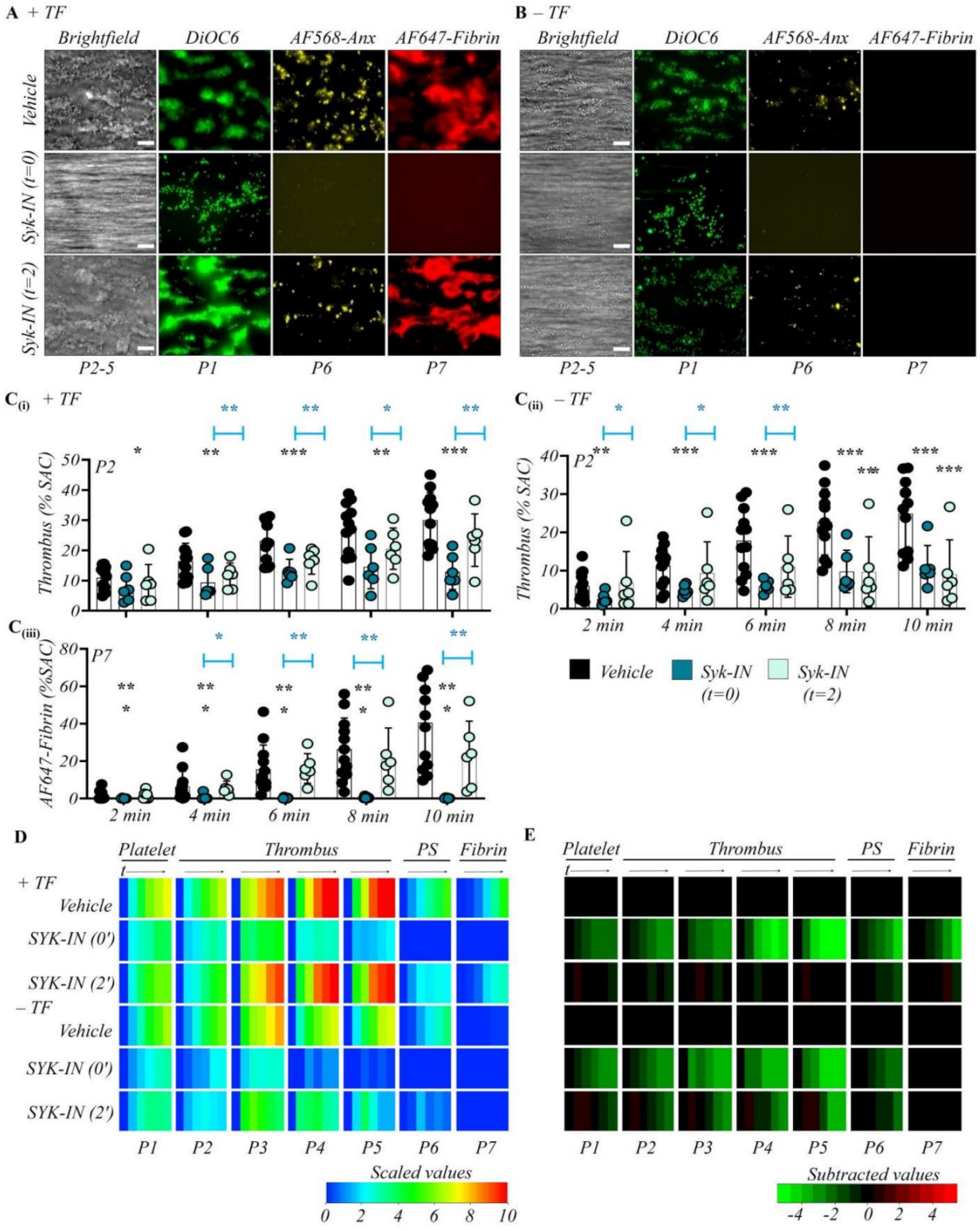


Figure 4. Time-confined role of Syk tyrosine kinase in thrombus formation. Citrated whole blood was labeled and co-perfused with recalcification medium over collagen and collagen/TF microspots (n = 6), as for Figure 1. Where indicated, perfusion was switched from control blood to blood preincubated with vehicle or Syk-IN (PRT-060318, 20 µM). Thrombus parameter analysis and heatmap presentation were as for Figure 1. (**A**, **B**) Representative end stage images of vehicle control condition; early Syk inhibition (Syk-IN from start); later Syk inhibition (Syk-IN from 2 min).

◀Images from microspots without (**A**) or with (**B**) TF. Quantitative analysis from ± TF surfaces of parameter P2: thrombus coverage (**C i**, **C ii**), and P7: fibrin deposition (**C iii**). Means ± SD, *p <0.05, **p <0.01, ***p <0.001 vs. indicated group (*t*-test). (**D**) Heatmap of univariate scaled time-dependent values per parameter (P1–7) for indicated surfaces and conditions. (**E**) Subtracted heatmap of scaled parameters versus collagen control runs. Color codes as indicated in color bars. For additional data, see Suppl. Figure 4.

Interestingly, the 2 min introduction of Syk-IN did no longer affect the thrombus parameters on collagen/TF microspots. On the other hand, for collagen-only microspots, platelet deposition (P1), the thrombus characteristics (P2–5), and phosphatidylserine exposure (P6) were still reduced at some time points (Figure 4). To sum up, these data confirmed a key role of the GPVI-ITAM signaling pathway involving Syk in mostly the early phase of collagen-induced thrombus formation, whose role extended to later time points only in the absence of TF.

Continued requirement of integrin αIIbβ3 activation in formation of platelet-fibrin thrombi

Considering that integrin αIIbβ3-mediated platelet aggregation is an essentially reversible event,[38] we then monitored the effects of early and late inhibition of αIIbβ3 activation using the integrin antagonist tirofiban (1 µg/ml). As expected, the tirofiban intervention from start completely abrogated the assembly of multi-layered platelet thrombi on collagen microspots, both in the presence and absence of TF (Figure 5A-B). Yet, a monolayer of adhered and spread platelets still formed on either surface type, which illustrated the central role of integrin αIIbβ3 in thrombus build-up but not in flow-dependent platelet adhesion. On collagen/TF surfaces, early tirofiban greatly decreased the thrombus parameters, while phosphatidylserine exposure stayed unaltered and fibrin formation reduced incompletely (Figure 5C-E). On collagen-only surfaces, tirofiban induced similar changes, although it

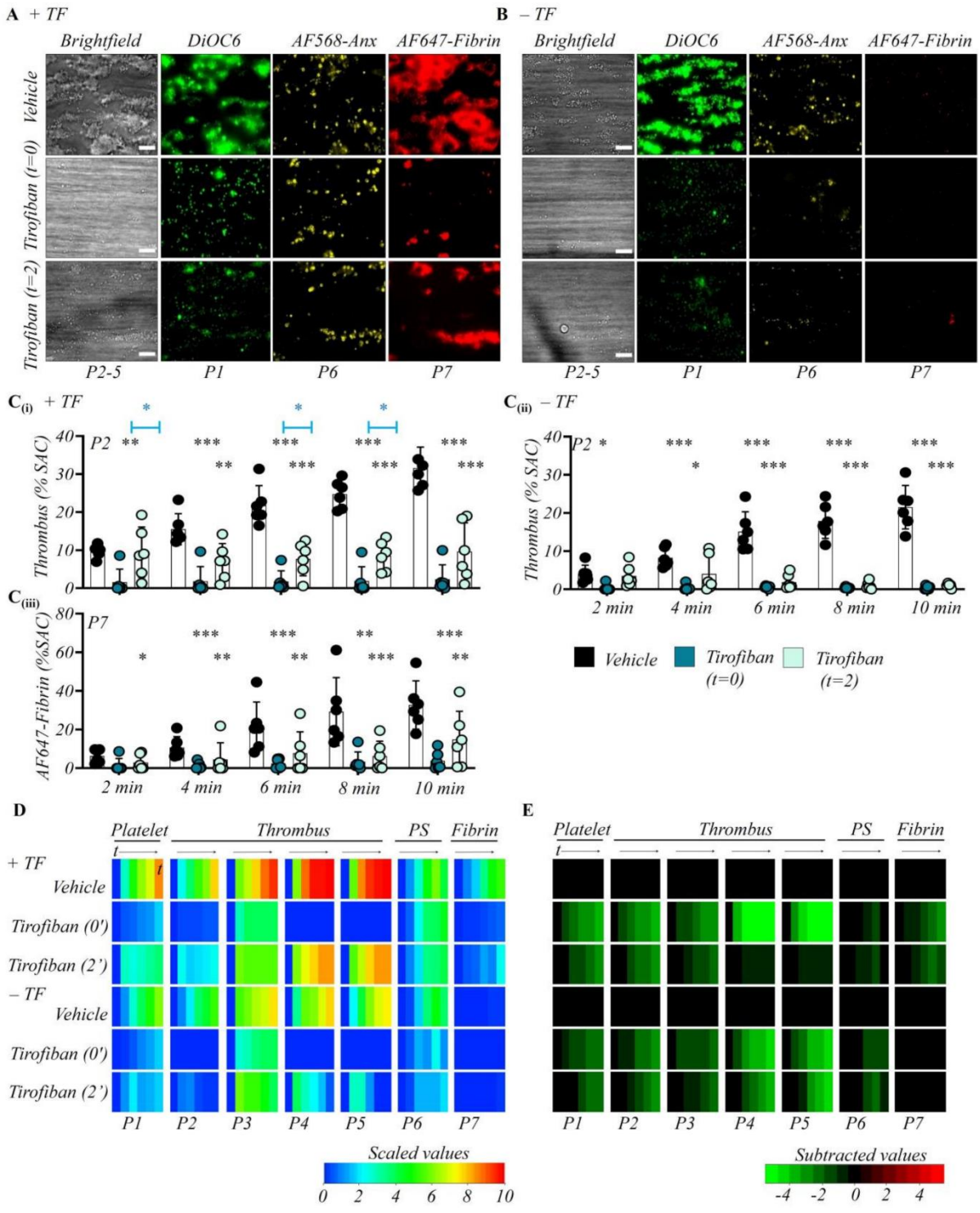


Figure 5. Involvement of integrin αIIbβ3 in thrombus formation independently of tissue factor. Citrated whole blood was labeled and co-perfused with recalcification medium over collagen and collagen/TF microspots (n = 6), as for Figure 1. Where indicated, perfusion was switched from control blood to blood preincubated with vehicle or integrin αIIbβ3 inhibitor (tirofiban, 1 µg/ml). Thrombus parameter analysis and heatmap presentation were as for Figure 1. (**A**, **B**) Representative end stage images of (i) vehicle control condition; (ii) early integrin inhibition (tirofiban from start); (iii) later integrin inhibition (tirofiban from 2 min). Images from microspots without (**A**) or with (**B**) TF; bars = 20 µm. Quantitative analysis from ± TF surfaces of parameter

◄P2: thrombus coverage (**C i**, **C ii**), and P7: fibrin deposition (**C iii**). Means ± SD, *p <0.05, **p <0.01, ***p <0.001 vs. indicated groups (t-test). (**D**) Heatmap of univariate scaled time-dependent values per parameter (P1-7) for indicated surfaces and conditions. (**E**) Subtracted heatmap of scaled parameters versus collagen control runs. Color codes as indicated in color bars. For additional data, see Suppl. Figure 5.

caused a small reduction in phosphatidylserine exposure at 6–8 min, which was related to the lower number of adhered platelets (Suppl. Figure 5). Interestingly, the addition of tirofiban after 2 min of blood perfusion led to dismantlement of the thrombi that were formed earlier on the collagen-only surfaces. Multi-layered platelet thrombi were completely gone at end-stage (Figure 5B, C), thus pointing to a retroactive tirofiban effect. On the other hand, on collagen/TF surfaces, the pre-formed thrombi remained intact, but the further thrombus build-up was prevented (Figure 5A, C). In addition, with late tirofiban phosphatidylserine exposure was not affected and fibrin clots were still forming (Figure 5D-E). To sum up, the integrin αIIbβ3 antagonist antagonized the process of thrombus growth from the time point of intervention, and even reversed this process if TF was absent.

Discussion

In the present study, we used a novel in-house developed intervention method for acutely switching (inhibited) blood samples during perfusion through a microfluidic chamber in order to resolve the time-dependency of platelet activation and coagulation processes via collagen (GPVI-induced ITAM and Syk tyrosine kinase signaling) and TF/thrombin (via factor VIIa and PAR1/4 receptors). As summarized in Figure 6, the results show that all the investigated pathways were crucial for the formation of platelet-fibrin thrombi during the first 2 min of flow. On the other hand, only platelet activation via PAR1/4 and integrin αIIbβ3 activation contributed at later time points. Given that PAR4[39] and of GPVI[40] have been

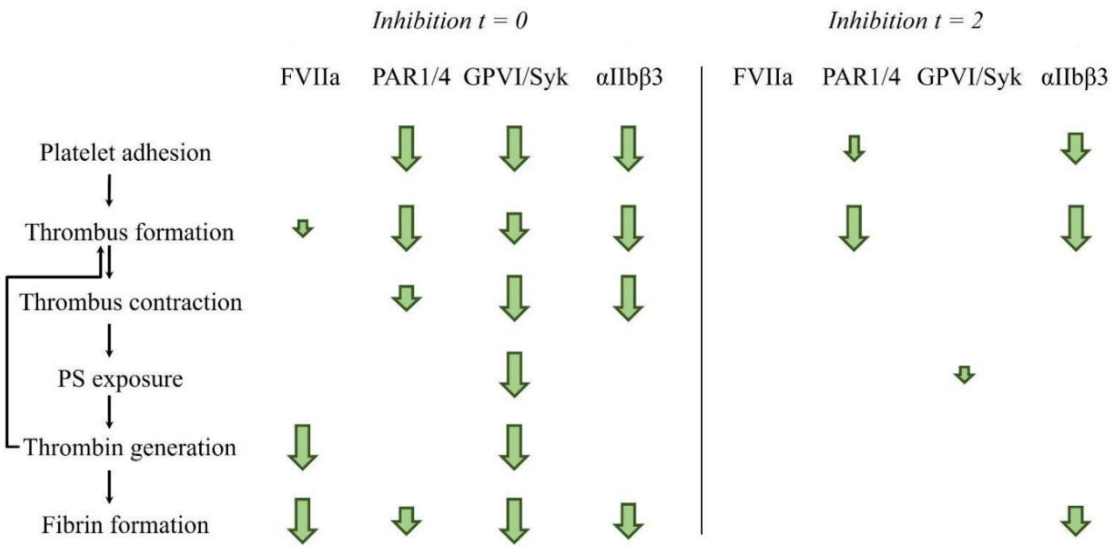


Figure 6. Schematic overview of early and late contribution of platelet and coagulation pathways in thrombus formation on collagen/TF surfaces under flow. Arrow sizes show to which extent inhibition of FVIIa, PAR1/4 receptors, GPVI signaling through Syk kinase, or integrin αIIbβ3 affect the early (0–2 min) and late (2–10 min) stages of the thrombotic process.

considered as possible targets for antithrombotic therapy, our results suggest that especially in case of GPVI-dependency an early intervention is important.

Previously, we have demonstrated that the current multiparameter approach of whole-blood thrombus formation under flow over collagen- and TF-coated surfaces provides detailed insights into the importance of specific platelet and coagulation pathways, and that this method can be used as a proxy assessment for hemostasis to explain the bleeding phenotypes of patients with certain platelet or coagulation defects.[10,26,41] In addition, we and others established that collagen-based microfluidic tests can be predictive for the outcome of *in vivo* models of hemostasis and thrombosis with genetically modified mice.[25,42,43] This background strongly supported us to adapt the microfluidic technology for obtaining information on the time dependency of key pathways of the platelet activation and thrombus-clot formation. For that purpose, we changed the flow perfusion method in a way that pharmacologically inhibited blood samples could enter the microfluidic chamber at a precisely chosen time point. As a suitable interval for separating ‘early’ and ‘late’ events

at arterial shear rate of 1000 s^{-1}, we choose 2 min, *i.e.*, a time point where the first multi-layered platelet aggregates are formed on collagen-like surfaces.[26] The present results show that separating the process into the first 2 min and later provides good insight into the earlier and later ways of platelet activation.

In the past decades, extensive research has revealed the roles of multiple platelet receptors in the flow-dependent reactions of platelet tethering, adhesion, secretion, aggregation, and coagulant activity (reviewed in Refs.[8,9,44,45]). *In vivo* findings have identified intricate interactions between (TF-induced) thrombin generation and platelet responses to arterial thrombus and clot formation.[4,5] Using the present, modified in vitro flow assay, we can now establish that the roles of GPVI and Syk signaling are most important at earlier stages, such in contrast the roles of platelet thrombin receptors and integrin αIIbβ3, which remained active during a more prolonged time. The present findings are in agreement with studies that in mice the lack of GPVI, obtained via genetic knockout or antibody-mediated depletion, partly protecting from arterial thrombus formation, leaving the generation of unstable, non-contracted thrombi.[46,47] The antithrombotic effects of inhibition of the human platelet PAR1 and PAR4 receptors has also been noticed.[48,49] Already two decades ago, in mice, the deficiency of PAR3 or PAR4 was found to have a thrombo-protective effect.[50] This was also shown for the PAR4 antagonist BMS-986120 when injected into cynomolgus monkeys.[39] These earlier studies hence fit with the current data obtained with pre-incubated blood samples. However, these studies were unsuited to inform on the later temporal roles of these receptors in arterial thrombus formation.

Our data with collagen/TF microspots pointed to an initial role of the extrinsic coagulation pathway via TF and factor VII in thrombin and fibrin formation, seemingly promoted by the procoagulant surface of phosphatidylserine-exposing platelets. This finding also agrees with the literature, underlining the role of platelet phosphatidylserine exposure in thrombus formation.[12] Indeed, the absence of this platelet response in the rare

human Scott syndrome leads to a mild bleeding disorder.[36] In atherothrombosis, the initiating role of the TF-factor VIIa pathway has been well studied.[51] In mice, using an in vivo model of atherosclerotic plaque rupture, a study found that inhibition of factor VIIa reduced the arterial thrombus size at early stages.[52] However, whether the exposed TF had a time-restricted role was not investigated.

In recent years, fibrinogen and fibrin are also known as ligands for GPVI during thrombus growth.[30,53] Flow experiments pointed to a sustained but weak GPVI-induced signaling that mostly promoted thrombus stabilization.[31] Our present data suggest that a later, fibrin-dependent role of GPVI is yet smaller than the initial collagen-dependent role. Both GPVI- and PAR-mediated activation can have mutually stimulating effects on platelet activation processes.[54] It appeared that the 'memory' of platelets for GPVI-induced activation is longer than for PAR-induced activation. Such a difference in signaling length could also explain the presently identified longer contribution of PARs than of GPVI in the thrombus growth on collagen/TF surfaces. This would imply that the time-confined action of thrombin on platelets necessitates a continued cleavage and activation of these protease-activated receptors.

An interesting observation was the similarly ranged but slightly lower effect of GPVI blockage (with EMF-1 Fab) as compared to Syk inhibition (with Syk-IN). An additional effect of Syk-IN of thrombus formation can have two causes. On the one hand, the EMF-1 Fab cannot block the contribution of other platelet receptors that signal via the Syk kinase, *i.e.*, CLEC-2 and FcγRIIa. A role of CLEC-2 in murine thrombus formation has been postulated.[55] This seemed to be redundant to that of GPVI, as double-deficient mice showed an increased protection from thrombosis and incremented bleeding risk.[56] Since no tool is available to block human CLEC-2, we could not investigate this possibility. On the other hand, we cannot exclude that the EMF-1 Fab still allowed residual GPVI action via a second epitope for collagen binding.[57]

A prominent finding was the continued and leading contribution of integrin αIIbβ3 activation (inhibited by tirofiban) to thrombus growth, multilayering, and contraction. This points to a regulatory contribution of αIIbβ3-mediated platelet–platelet contacts during the whole period of thrombus formation and stabilization. Notably, with tirofiban present, the ability of platelets to expose phosphatidylserine was unaltered, albeit this response was reduced in absence of TF due to a lower platelet adhesion. On collagen-only surfaces, we noted that tirofiban but not GPVI blockage had a retroactive effect by inducing destabilization of the formed platelet aggregates. The destabilizing effect was typically lost in the presence of TF, which can be explained by a counter-effect of early formed cross-linked fibrin fibers in the thrombi.[20] Our findings thus mirror the antithrombotic *in vivo* capacity of αIIbβ3 inhibition by tirofiban and other integrin antagonists.[22]

Materials and Methods

Materials

Recombinant human TF was purchased from Dade-Behring (Breda, The Netherlands), and collagen type I (Horm) was purchased from Takeda (Hoofddorp, The Netherlands). Alexa Fluor (AF)568-labeled annexin A5 was obtained from Life Technology (Carlsbad, CA, USA). The platelet probe 3,3′dihexyloxa carbocyanine iodide ($DiOC_6$) came from AnaSpec (Fremont, CA, USA); AF647-labeled human fibrinogen was obtained from Molecular Probes (Eugene, OR, USA). PAR1 inhibitor atopaxar hydrobromide (E5555) was obtained from Axon Medchem (Groningen, The Netherlands). The PAR4 inhibitor BMS-986120 was obtained from Cayman Chemical (Ann Arbor, MO, USA). The anti-human GPVI Fab fragment EMF-1 was generated by Emfret Analytics Eibelstadt/Würzburg, Germany (unpublished data September 2021). The selective Syk inhibitor PRT-060318 (Syk-IN) came from Bio-Connect (Huissen, The Netherlands). The integrin αIIbβ3 inhibitor tirofiban and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (St. Louis, MI, USA).

Pluronic was obtained from Invitrogen (Carlsbad, CA, USA). Other materials came from previously described sources.[26,58]

Blood donors and blood collection

Blood was donated by healthy volunteers that were free from anticoagulant or antiplatelet medication for a minimum period of 4 weeks. Studies were approved by the local Medical Ethics Committees (Maastricht University Medical Centre^{+}). All donors provided full informed consent in accordance with the Declaration of Helsinki, and procedures were in accordance with the local regulations and guidelines. Blood drawing was by venipuncture using a vacuum container. The blood was collected into a 9 ml tubes with 3.2% trisodium citrate (Greiner, Alphen a/d Rijn, The Netherlands). For all the microfluidics studies, collected blood was stored at room temperature and used within 4 h.

Preparation of microspot coatings

Glass coverslips (24 × 60 mm, Thermo-Fisher, Breda, the Netherlands) were cleaned and degreased and subsequently freshly coated with the help of a precision mall with collagen as two adjacent microspots (≈1 mm of diameter and 5 mm center-to-center distance), of which the downstream spot contained TF, as detailed elsewhere.[26] To prevent a cross-over effects, we placed the most thrombogenic microspot (collagen/TF) as second in the direction of blood flow. Coating was performed with 1 µl of collagen type I (50 µg/ml), and, after 1 h with extra 1 µl TF (500 pM), including a washing step in between. After another incubation with Hepes buffer (pH 7.45; 2 mM $MgCl_2$, 0.1% glucose, 10 mM Hepes, 136 mM NaCl, and 2.7 mM KCl), the coverslips were blocked using Hepes buffer containing 1% BSA (w/v). Subsequently, these were mounted on a transparent flow chamber (50 µm height, 3.0 mm width and 30 mm length) and pre-rinsed with Hepes buffer at a pH of 7.45 with 0.1% BSA added.[20]

Recalcification and mixing of blood samples under flow in microfluidic chambers

Citrate-anticoagulated blood samples were in situ recalcified while perfusing through the flow chamber under conditions of full blood-buffer mixing, essentially as described before,[26] but using a three-way mixing tube system (Suppl. Figure 1). Two citrated blood samples in 1.0 mL plastic syringes were connected to two of the tube inlets and sequentially perfused through the flow chamber using pulse-free micro-pumps (Model 11Plus, 70–2212, Harvard Apparatus, Holliston, MA, USA). The third tube inlet was connected to a 1 ml syringe containing recalcification medium with 63 mM $CaCl_2$ and 32 mM $MgCl_2$ in Hepes buffer with a pH of 7.45 (Suppl. Figure 6). Mixing of either blood sample with recalcification medium was at a volume ratio of 10:1. Flow rates were set to provide a final calculated final wall-shear rate of 1000 s^{-1}.[59] Blood samples for the secondary perfusion were pre-incubated with indicated inhibitor for 10 min at room temperature. As a standard procedure, both the primary and secondary blood samples were pre-labeled with final concentrations of 0.5 µg/ml $DiOC_6$ (staining platelets), 8.5 µg/ml AF647-fibrinogen (staining of fibrin accumulation), and 4.0 µg/ml AF568-annexin A5 (staining phosphatidylserine-exposing platelets). Note that discriminative staining for detection strongly labeled fibrin vs. low-labeled fibrinogen was by threshold settings, as detailed elsewhere.[20] To facilitate the access of Syk-IN into the platelets, we pre-mixed the compound PRT-060318 in DMSO with pluronic (40 µg/ml), and then it was added to the blood.[37] The concentration of DMSO was kept at <0.5%. Per blood sample and condition, duplicate or triplicate (in case of variation) flow runs were performed.

Complete mixing of the blood samples with the medium for re-calcification was achieved by a two-phased mix procedure.[26] First, a three-way-shaped Versitec silicone tubing (1.0 mm ID, 3.0 mm OD, Saint-Gobain Plastics, France), fabricated in house, was built by a dual cross-wise needle insertion into the middle of a long portion of tube (at opposite sides). Two other tubes were then glued onto the central tube while removing the

needles. This created face-to-face openings in the middle tube, which allowed a free passage of fluids. Liquid polymerizing silicone was used to provide leakage-free sealing. The second phase of mixing occurred when the central tube outlet entered the flow chamber inlet, which resulted in the rapid transition from a tubular (1 mm Ø) to a flat (width 3 mm and height 50 µm) cross-section, and consequently in an acute redistribution of the flow velocity profile.[26] This set up ensured the continuous and consistent mixing with a low shear rate at the inlet, while keeping a laminar shear flow inside of the parallel-plate chamber. Full mixing was visually inspected by microscopy (absence of separate streams of blood and recalcification medium).

By usage of two connected syringes with blood, it was possible to initially perfuse an untreated blood sample (2 min) and then to switch to a treated blood sample, with the third syringe pump with constant running of recalcification buffer. This operation procedure allowed for an immediate switch from untreated to treated blood without stasis, and preventing mixing of the blood samples. All flow runs were performed with the three-tube system.

Microscopic real-time detection of multicolor thrombus formation on microspots

Microscopic brightfield and three-color overlay imaging of thrombus formation on two microspots was performed, essentially as described.[26] In brief, fluorescence and brightfield microscopic images were acquired by rapid switching of dichroic cubes [brightfield, or filter sets with excitation wavelengths 626 nm (Cy5), 531 nm (RFP), and 470 nm (GFP)]. An inverted EVOS fluorescence microscope was used to record images (Life Technology, Ledeberg, Belgium). The microscope was equipped with an Olympus 60× oil-immersion objective with high *z*-axis resolution (UPLSAPO60, numerical aperture 1.35). The images were collected as 8-bit monochromes by a sensitive camera, providing 1360 × 1024 pixels

and a resolution of 0.108 µm per pixel. Per flow run, image sets were collected at t = 2, 4, 6, 8, and 10 min.

Standardized microscopic image analysis and assessment of thrombus parameters

Brightfield and fluorescence images were analyzed with the help of semi-automated scripts, designed in the open-access program Fiji.[60] Specific scripts were designed for brightfield images and for each fluorescent label, essentially as described.[61,62] In these scripts, optimized fast Fourier transformation was applied to filter down the image-wide structures and reduce background noise. Afterwards, morphological horizontal and vertical erode and dilate steps were applied to remove the noise and to enhance relevant structures. In order to verify the binary masked images, overlay images were generated. In case of incorrect overlays, scripts allowed to loop back for the resetting of thresholds. Per microspot and time point, the analyzed fluorescence and brightfield images resulted in seven parameters (P1–7) (Table 1). Fluorescence $DiOC_6$ images reported on platelet deposition (P1), and enhanced brightfield images showed thrombus surface area coverage (P2). Furthermore, brightfield images were scored for thrombus morphology (P3): 0, no or little attached platelets; 1, multiple single attached platelets; 2, widespread coverage of single attached platelets; 3, small platelet aggregates; 4, medium-sized aggregates or thrombi; 5, big aggregates or thrombi.[61] In addition, scoring (scale 0–3) for thrombus contraction (P4) and thrombus multilayering (P5). Other fluorescence images provided quantitative information on phosphatidylserine-exposing platelets (P6, AF568-annexin A5); furthermore, on fibrin formation (P7, thresholded AF647-fibrinogen).

Data handling and statistics

Heatmaps were generated using the program R. For heatmap representation, all parameter values were univariate-normalized to a range of 0–10.[61] To obtain one parameter set per

microspot and donor, we averaged thrombus parameters values of duplicate or triplicate flow runs from the same blood donor. In order to visualize treatment effect, we linearly subtracted scaled parameters in order to obtain subtraction heatmaps. For each inhibition time-point (t = 0 and t = 2 min), differences between mean values of control and inhibitor runs were determined between treated and untreated (vehicle control) blood samples per donor using a paired Student's *t*-test. P-values below 0.05 were considered to be significant. In addition, for the subtraction heatmaps, a filter of $p < 0.05$ was added to visualize relevant effects where indicated. Numerical data of interventions are presented as means with SD.

Conclusions

In conclusion, this study presents a new efficient adaptation of the Maastricht microfluidic system, allowing disclosure of the time-dependent roles of key pathways of platelet activation and coagulation in the thrombus development. Our first-time study on the interference in thrombus formation at different time points has revealed typical differences between early and late inhibition (Figure 6). Our data indicate that, in the microfluidic setting, GPVI-induced platelet signaling is pivotal only in the early phases of thrombus growth, although this signaling can continue to induce phosphatidylserine exposure in later phases. Furthermore, it appears that thrombin induction via PAR1/4 continues to be functionally active in thrombus growth for a longer time-period as compared to GPVI-induced signaling. Finally, integrin αIIbβ3 activity is operative during all phases of thrombus formation. The present results thereby provide a significant addition to the current understanding of the sequence of molecular processes in arterial thrombus formation.

Author Contributions

S.N. performed the experiments, analyzed the data, and wrote the manuscript. D.S. provided supervision. B.N. provided supervision and provided the anti-hGPVI antibody. J.W.M.H. and M.J.E.K. provided expert supervision for the flow-adhesion assay and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding

S.N. is supported by the European Union's Horizon 2020 research and innovation program under the Marie Slodowska-Curie grant agreement no. 766118 TAPAS and is enrolled in a joint PhD program at the Universities of Maastricht (the Netherlands) and Würzburg (Germany).

Institutional Review Board Statement

The study was approved by the local Medical Ethics Committees (Maastricht University Medical Centre^{+}, NL31480.068.10). All subjects provided full informed consent according to the Declaration of Helsinki, and all methods were performed in accordance with the relevant guidelines and regulations.

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study. According to ethical permission, all subjects provided blood without tracing to individuals.

Data Availability Statement

All data are included in the manuscript as figures, tables, or supplementary figures.

Conflicts of Interest

J.W.M.H. is co-founder and shareholder of FlowChamber. The other authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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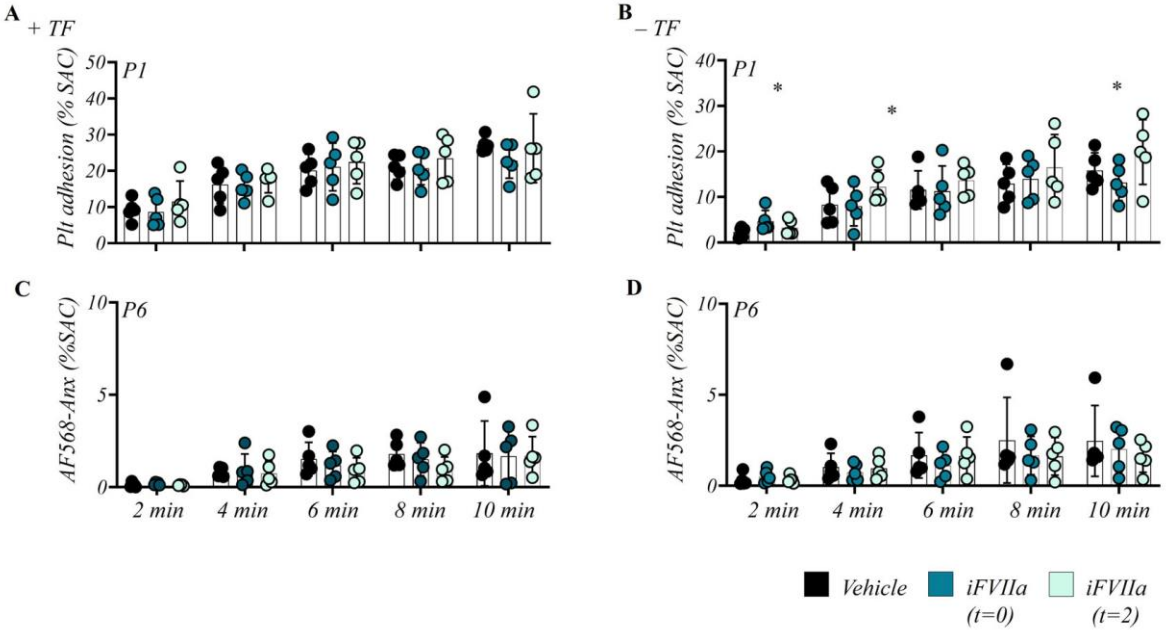
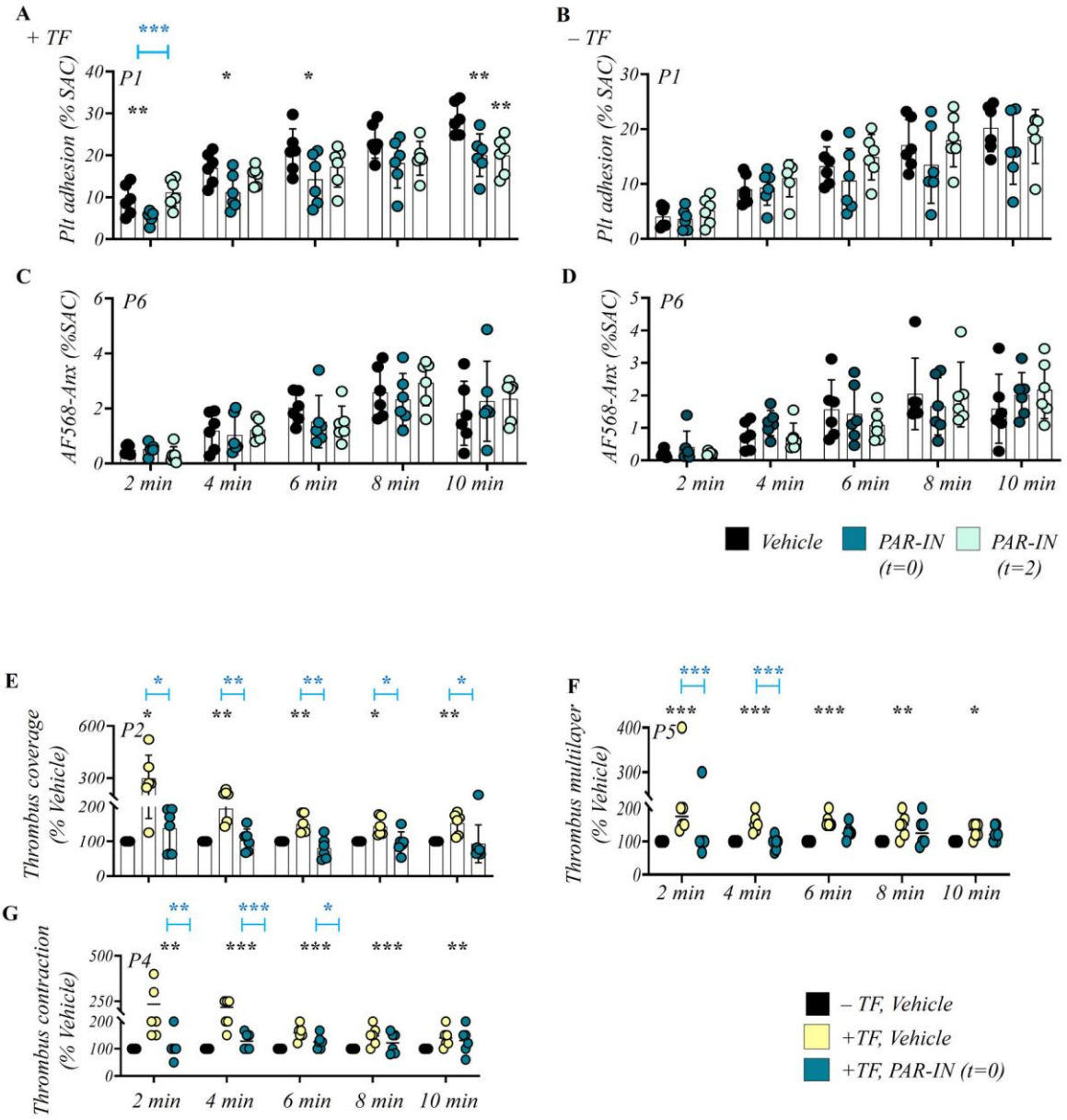
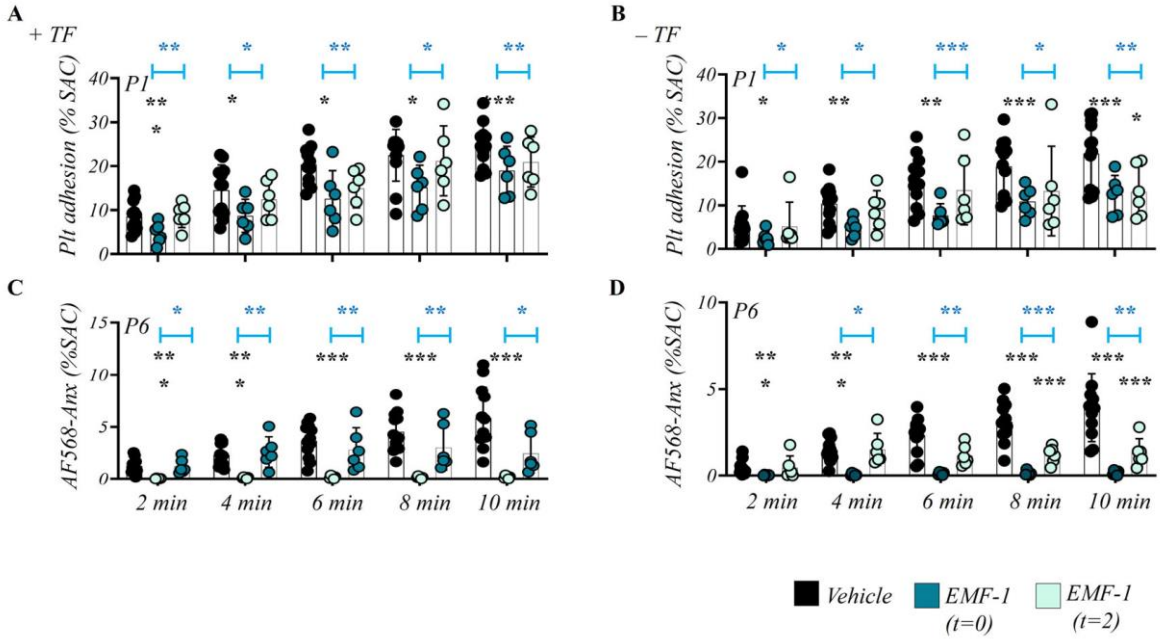
Supplemental Figures to Chapter 3



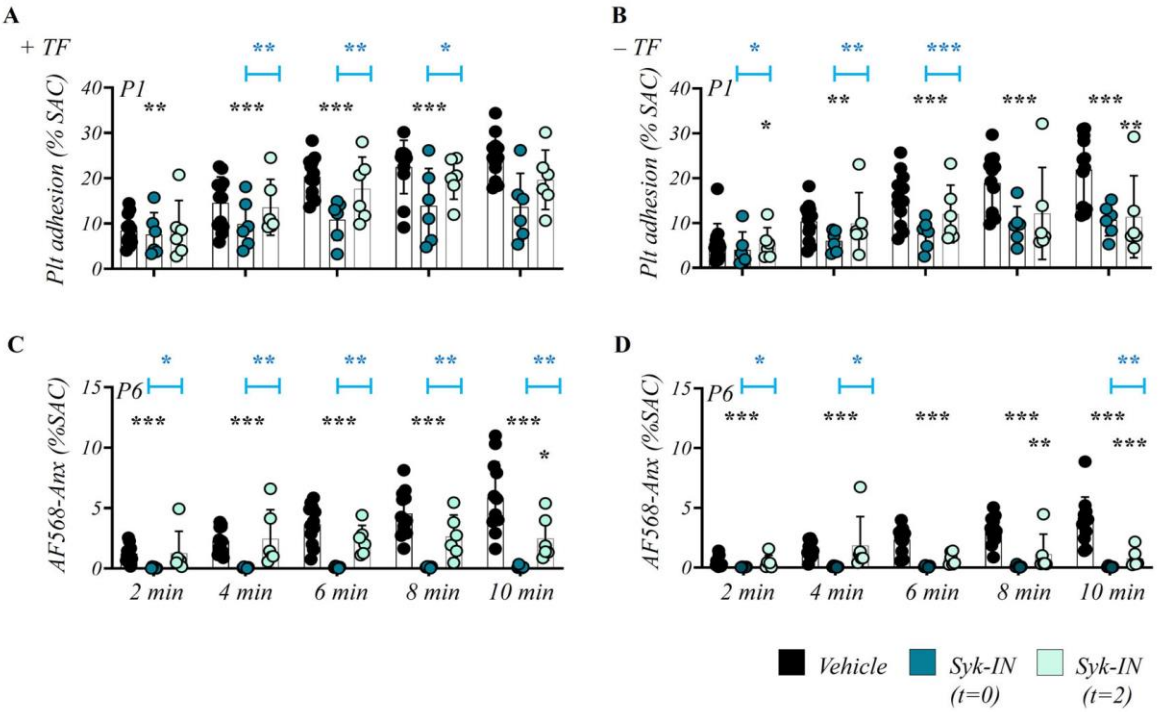
Suppl. Figure 1. Priming role of TF in progression of platelet-fibrin thrombus formation on collagen under high shear. Citrated whole blood from healthy subjects (n = 5) was supplemented with fluorescent labels to simultaneously detect platelet adhesion (P1, DiOC6), thrombus and platelet multilayer characteristics (P2-5, brightfield), phosphatidylserine exposure (P6, AF568-annexin A5) and fibrin deposition (P7, AF647-fibrin). Where indicated, perfusion was from start (t = 0) with iFVIIa-treated blood (1 µM, f.c.), or the control blood was switched after 2 min with iFVIIa-treated blood (t = 2). Control blood runs were carried out with vehicle solution. During blood flow, monochromatic images in 4 colors were captured from collagen/TF and collagen microspots by microscopy at 2, 4, 6, 8 and 10 min. Shown are quantitative effects of iFVIIa on thrombus parameters: (**A-B**) platelet adhesion (P1); and (**C, D**) AF568-annexin A5 staining for phosphatidylserine exposure (P6). Means ± SD, *p <0.05, **p <0.01, ***p <0.001 vs. indicated group (t-test). See further Figure 1.



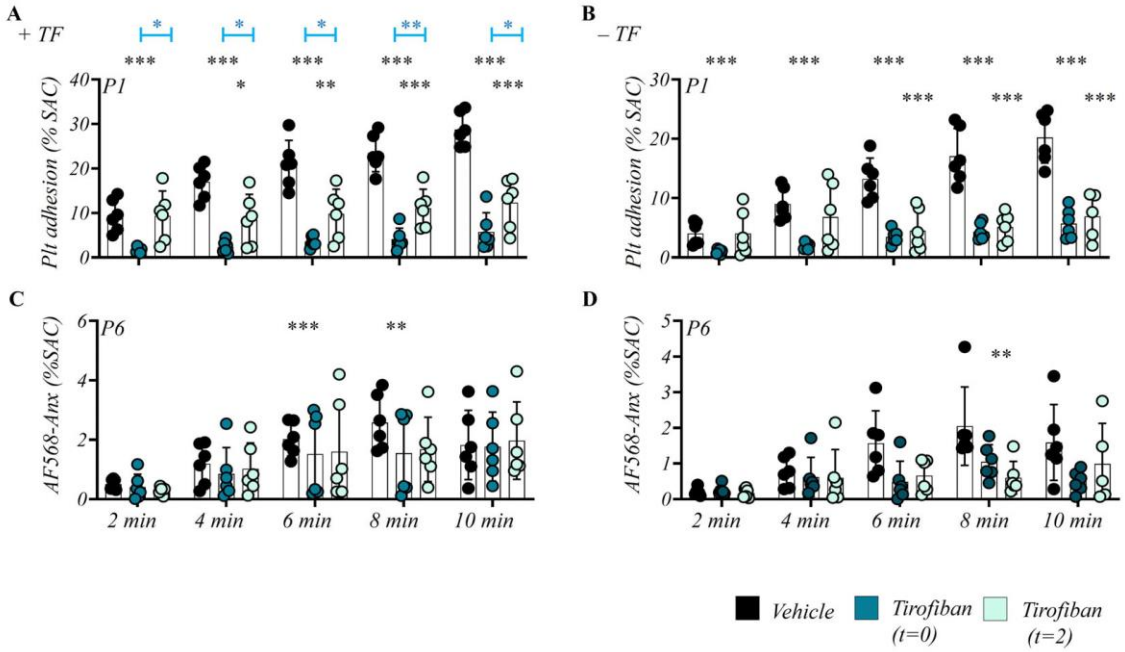
Suppl. Figure 2. Temporal roles of platelet thrombin receptors PAR1 and PAR4 in platelet-fibrin thrombus formation on collagen with TF. Citrated whole blood samples from healthy subjects (n = 6) were supplemented with fluorescent labels, and perfused over microspots of collagen and collagen/TF, as described for Figure 1. Where indicated (PARIN), perfusion was switched from control blood to blood preincubated with vehicle or a mix of atopaxar (PAR1 inhibitor, 2 µM, f.c.) and BMS-986120 (PAR4 inhibitor, 1 µM, f.c.). Shown are quantitative effects of PAR-IN on thrombus parameters: (**A, B**) platelet adhesion (P1); and (**C, D**) AF568-annexin A5 staining for phosphatidylserine exposure (P6). Furthermore, normalized (vs. -TF vehicle) quantification per donor of effects of +TF vehicle and PAR-IN on: (**E**) thrombus coverage (P2); (**F**) thrombus multilayering (P5); and (**G**) thrombus contraction (P4). Means ± SD, *p <0.05, **p <0.01, ***p <0.001. vs. indicated group (t-test). See further Figure 2.



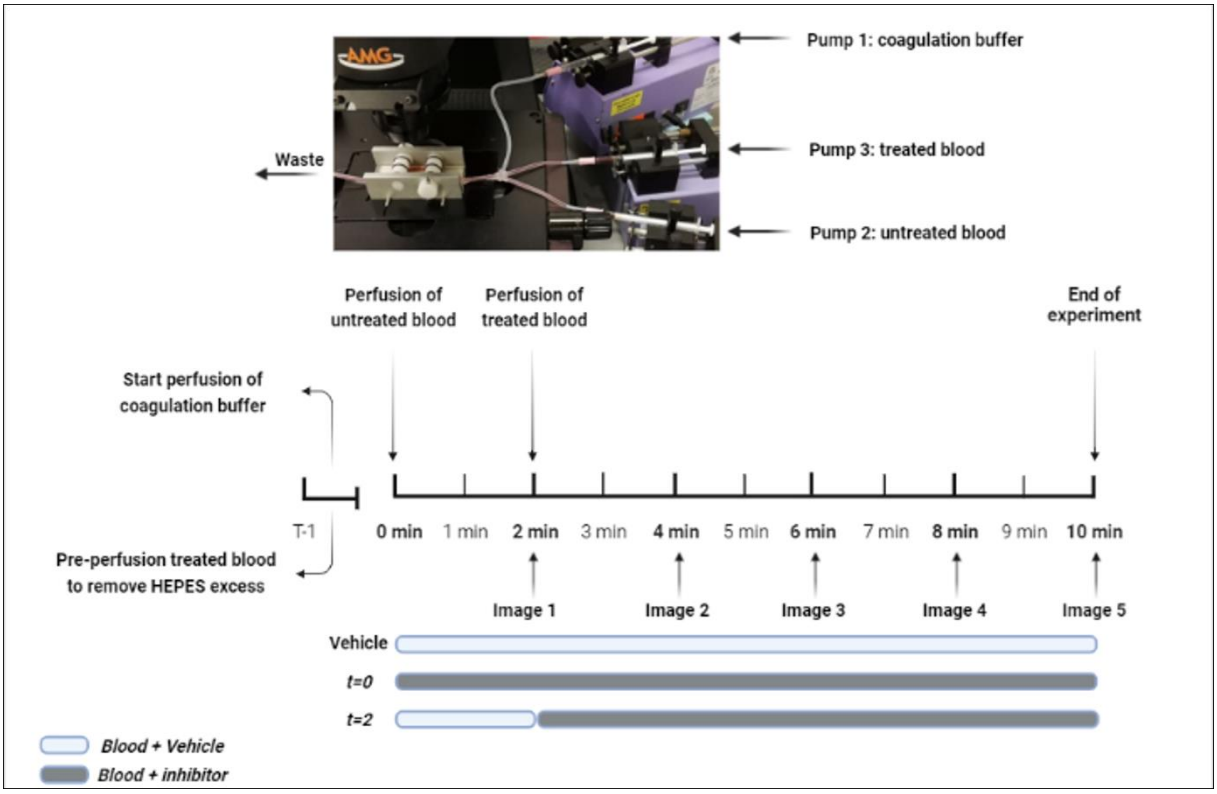
Suppl. Figure 3. Time-dependent role of GPVI receptor in platelet-fibrin thrombus formation on collagen. Citrated whole blood was labeled and co-perfused with recalcification medium over collagen and collagen/TF microspots (n = 6), as for Figure 1. Where indicated, perfusion was switched at 2 min from control blood to blood preincubated with vehicle or anti-GPVI Fab EMF-1 (10 µg/ml, f.c.). Shown are quantitative effects of EMF-1 Fab on thrombus parameters: (**A-B**) platelet adhesion (P1); and (**C-D**) AF568-annexin A5 staining for phosphatidylserine exposure (P6). Means ± SD, *p <0.05, **p <0.01, ***p <0.001 vs. indicated group (t-test). See further Figure 3.



Suppl. Figure 4. Temporal role of platelet Syk activation in platelet-fibrin thrombus formation independently of TF. Citrated whole blood was labeled and co-perfused with recalcification medium over collagen and collagen/TF microspots(n = 6), as for Figure 1. Where indicated, perfusion was switched at 2 min from control blood to blood preincubated with vehicle or Syk-IN (PRT-060318, 20 µM). Shown are quantitative effects of Syk-IN on thrombus parameters: (**A-B**) platelet adhesion (P1); and (**C-D**) AF568-annexin A5 staining for phosphatidylserine exposure (P6). Means ± SD, *p <0.05, **p <0.01, ***p <0.001 vs. indicated group (t-test). See further Figure 4.



Suppl. Figure 5. Persistent role of integrin αIIbβ3 in platelet-fibrin thrombus formation independently of tissue factor. Citrated whole blood was labeled and co-perfused with recalcification medium over collagen and collagen/TF microspots (n = 6), as for Figure 1. Where indicated, perfusion was switched from control blood to blood preincubated with vehicle or integrin αIIbβ3 inhibitor (tirofiban, 1 µg/ml). Shown are quantitative effects of tirofiban on thrombus parameters: (**A-B**) platelet adhesion (P1); and (**C-D**) AF568-annexin A5 staining for phosphatidylserine exposure (P6). Means ± SD, *p <0.05, **p <0.01, ***p <0.001 vs. indicated group (t-test). See further Figure 5.



Suppl. Figure 6. Setup and flow chart of three-way tubing inlet system to for immediate switching from vehicle control blood to inhibited blood. (**A**) Picture of three connected tubes, allowing pressure infusion of recalcification medium into respective blood samples. The syringe attached to pump 1 contains recalcification medium with 32 mM $MgCl_2$ and 63 mM $CaCl_2$ in Hepes buffer pH 7.45, the syringe attached to pump 2 contains blood labeled with $DiOC_6$, AF568-annexin A5 and AF647-fibrinogen and vehicle, and the syringe attached to pump 3 contains blood with the same labels plus required inhibitor. (**B**) Flowchart showing the sequence of actions during an experiment. Pump 1 operated constantly at 1x flow rate. Before start of the experiment, pump 3 was run at 9x flow rate to allow removal of Hepes buffer pH 7.45 from the connection tube. The pump 3 was stopped once the blood reached the connection point. Pump 2 was run at 9x flow rate from start during the first 75 s, after which pump 3 took over. Of note, the time required for blood from the connection point to the inlet of the flow chamber was timed at 45 s (final calculated wall-shear rate of 1000 s^{-1}). This timing thus allowed perfusion for the first 2 min of untreated blood and consecutively of treated blood for the next 8 min.

Chapter 4

Scaled or complete in vivo depletion of human GPVI as a safe strategy for anti-thrombotic therapy

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To be submitted

I performed experiments, analyzed data and wrote the manuscript; S.B performed experiments and contributed to the manuscript; D.S. supervised research and contributed to writing the manuscript; B.N. designed the study, conceived experiments, supervised research, analyzed data and wrote the manuscript.

Chapter 5

Antibody-mediated depletion of human CLEC-2 in a novel humanized mouse model

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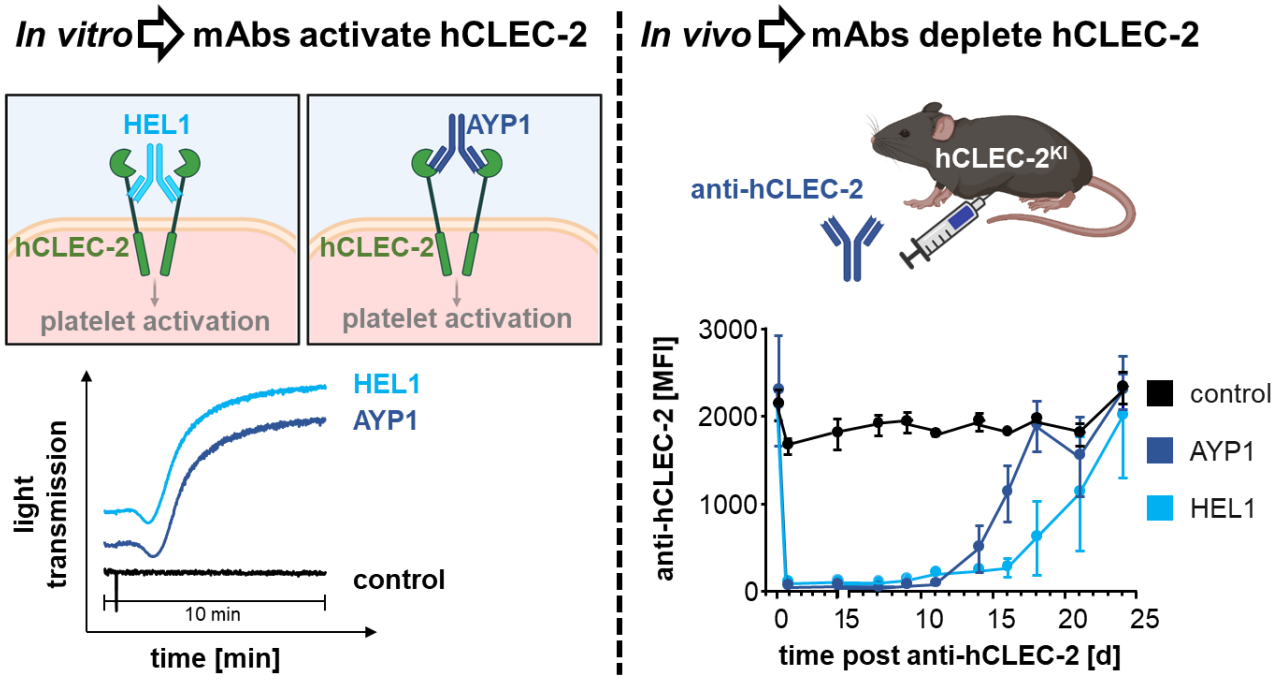
Blood Advances, 2023, in press

H.C.B. performed experiments, analyzed data and wrote the manuscript; S.B., E.M.S.J., J.K. and I performed experiments and analyzed data; Y.D. and V.M. provided vital reagents and proofread the manuscript; S.T., S.P.W. and B.N. supervised the research and edited the manuscript; D.S. supervised the research, performed experiments, analyzed the data and wrote the manuscript

Graphical abstract



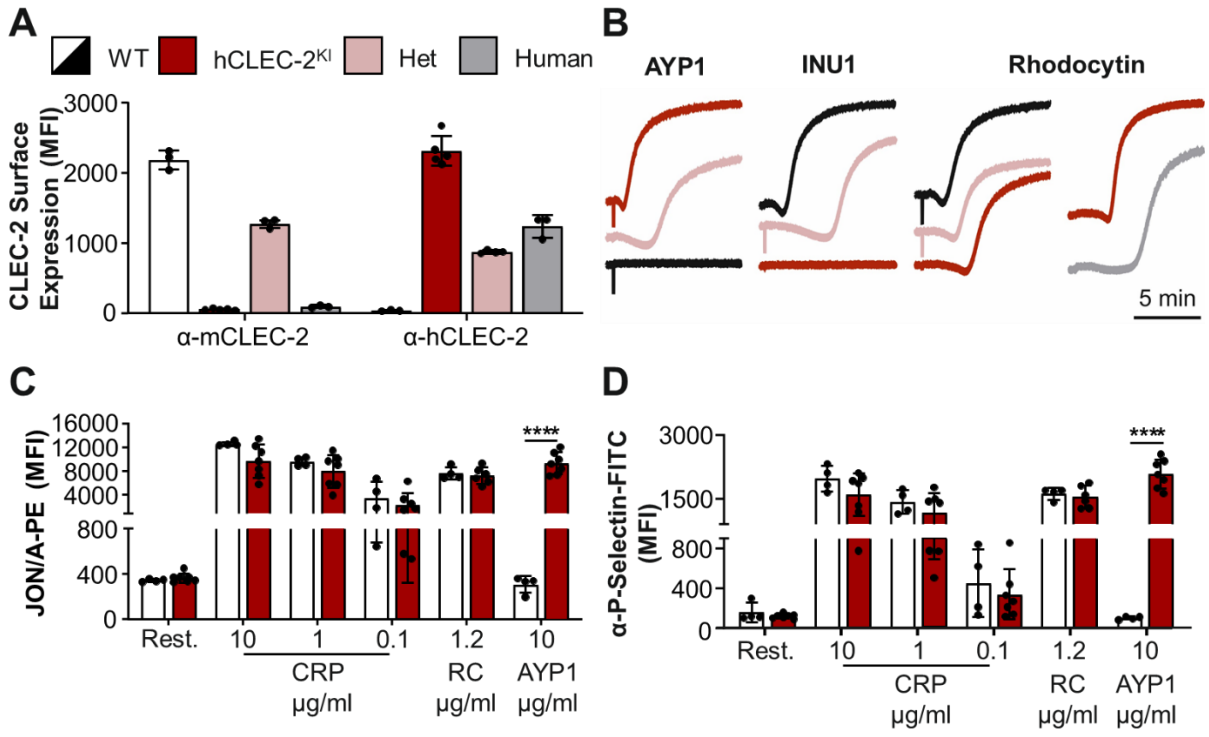
C-type lectin-like receptor 2 (CLEC-2) is a unique platelet activation receptor signaling through a single YXXL sequence, representing half of an immunoreceptor tyrosine-based activation motif (hemITAM).[1,2] CLEC-2 and its endogenous ligand podoplanin are crucial for normal development with mice deficient in either showing defective blood-lymphatic vessel separation.[3-5] This interaction also has a role in tumor metastasis.[6,7]

CLEC-2 is important in thrombosis, particularly in immuno-thrombosis and smaller vascular beds[2,8-12] with CLEC-2 deficiency reducing vessel occlusion in several in vivo thrombosis models with little effect on hemostasis.[13-15] Occlusion is unaltered in CLEC-2 Y7A signaling null mice, in which the receptor is normally expressed, suggesting it is the presence of CLEC-2 itself, rather than CLEC-2- induced platelet activation, that has a role in thrombus stability.[14] Furthermore, immuno-depletion of 48 CLEC-2 from the platelet surface using the monoclonal antibody INU1 has similar effects on thrombus formation, with depletion lasting up to 6 days and accompanied by transient thrombocytopenia.[8,13]

As a result, CLEC-2 has been suggested as a potential anti-thrombotic target. However, the *in vivo* role of human CLEC-2 cannot be readily investigated experimentally in humans, meaning there are limited methods to assess the relevance of human CLEC-2 in arterial thrombosis or tools to test potential therapeutics pre-clinically. In addition, although antibodies against human CLEC-2, such as AYP1,[16] exist it is unknown if human, like mouse, CLEC-2 can be immunodepleted. Here we have generated a humanized CLEC-2 mouse model that can be used to test potential anti-human CLEC-2 therapeutics *in vivo*.

Humanized CLEC-2 (hCLEC-2KI) mice were generated using CRISPR to replace the mouse gene with the human variant (Suppl. Figure 1). These mice are viable, fertile and born in Mendelian ratio (Suppl. Table 1). There were no obvious signs

of blood lymphatic defects and both platelet and megakaryocyte counts were comparable to wildtype (*WT*) mice (Suppl. Table 2, Suppl. Figure 2). This is in contrast to other mouse lines in which CLEC-2 or podoplanin have been genetically modified[3,4,14] and suggests that human CLEC-2 can compensate for loss of the 63 mouse protein and the interaction with murine podoplanin is sufficient for blood-lymphatic vessel separation. Human but not mouse CLEC-2 could be detected on platelets from hCLEC-2KI mice with heterozygotes expressing half each of human and mouse CLEC-2 (Figure 1A, Suppl. Figure 3). Surface abundance of CLEC-2 on hCLEC-2^{KI} platelets was approximately double that on human platelets (Figure 1A). The surface abundance of all other glycoprotein receptors was comparable to *WT* platelets (Suppl. Table 3) as were platelet activation and aggregation for G protein-coupled receptors (GPCR) as well as GPVI agonists (Figure 1B-D, Suppl. Figure 4). However, there was a slight increase in lag time prior to aggregation with rhodocytin, similar to that seen in human platelets (Figure 1B). Thrombus formation on collagen



◄ Figure 1. hCLEC-2KI mice have normal CLEC-2 expression and platelet activation. (**A**) Surface expression of mouse and human CLEC-2 by flow cytometry using INU1 and AYP1 antibodies respectively. Heterozygous mice expressed half human and half mouse CLEC-2 whereas hCLEC-2KI mice expressed only hCLEC-2 on their platelet surface. (**B**) Light transmission aggregometry with washed platelets shows that AYP1 (10 µg/ml) and INU1 (10 µg/ml) cause aggregation of hCLEC-2KI and WT platelets respectively and reduced aggregation in hCLEC-2KI heterozygous platelets. Rhodocytin (0.24 µg/ml) induced aggregation has a longer lag time in hCLEC-2KI platelets but also in human platelets. (**C**) Platelet integrin activation measured by JON/A-PE antibody binding in flow cytometry shows no difference in (hem)ITAM-mediated platelet activation in hCLEC-2KI platelets and hCLEC-2 specific activation by AYP1. (**D**) Platelet granule secretion measured using an anti-P-selectin antibody in flow cytometry was unaltered in hCLEC-2KI, following platelet activation by (hem)ITAM agonists, whereas AYP1 causes hCLEC-2 specific granule secretion. Data analyzed by two-way ANOVA followed by a Sidak's multiple comparison test, ****p <0.001. Results are shown as means ± SD with each circle representing one individual; results are representative of three independent experiments. *Abbreviations:* Het, heterozygous; MFI, mean fluorescent intensity; RC, rhodocytin; CRP, collagen-related peptide; PE, phycoerythrin; FITC, fluorescein isothiocyanate.

under flow at 1200 s^{-1} was unaltered in hCLEC-2^{KI} mice (Suppl. Figure 5). Platelet spreading was comparable to *WT* on both fibrinogen and CRP. However, it was slightly reduced on mouse podoplanin for hCLEC-2^{KI} platelets as, although they formed both filopodia and lamellipodia, there were few fully spread platelets (Suppl. Figure 6). Overall, this suggests that there are no major differences in platelet function in hCLEC-2^{KI} compared to *WT* mice.

The novel antibody HEL1 was generated by hybridoma technology following immunization of Wistar rats with hCLEC-2 immunoprecipitated from human platelet lysates. HEL1 is specific to hCLEC-2 and can be used in flow cytometry, western blotting and for immunoprecipitation (Suppl. Figure 7A-C). It binds to a different epitope on CLEC-2 than AYP1 as no competition between the two antibodies was observed (Suppl. Figure 7D), although both antibodies cause hCLEC-2^{KI} platelet aggregation

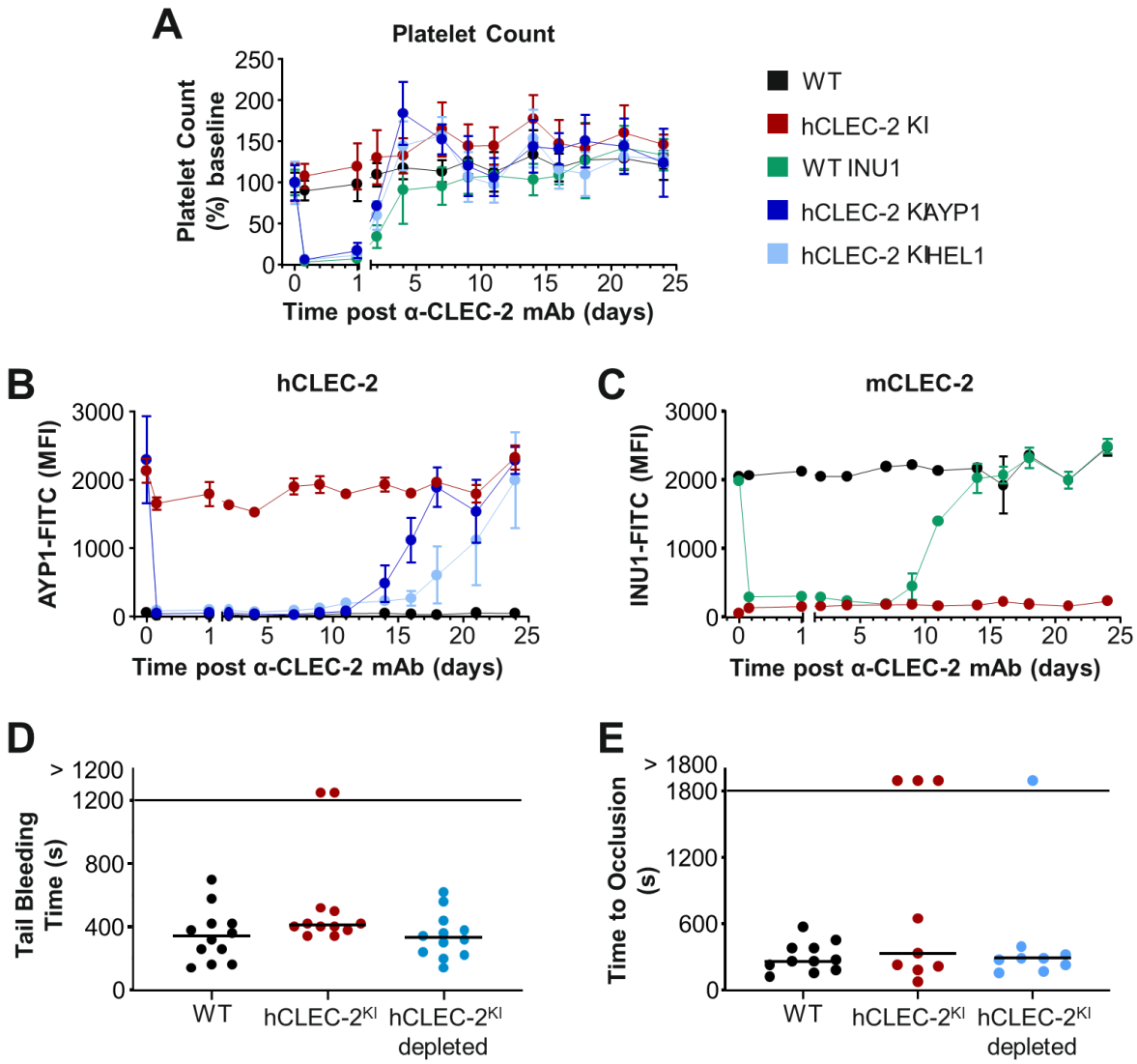


Figure 2. hCLEC-2 can be immunodepleted using HEL1 or AYP1 with little effect on hemostasis. (**A**) Platelet count following intraperitoneal injection of either INU1, AYP1 or HEL1 antibody (3 µg/g bodyweight). Transient thrombocytopenia lasting up to 4 days after injection can be seen for all antibody treated groups compared to the untreated controls. Platelet count was determined by flow cytometry and is shown as the percentage of the baseline count. (**B**) hCLEC-2 surface expression determined by flow cytometry following depletion by either AYP1 or HEL1. For both antibodies, CLEC-2 could not be detected on the platelet surface for at least 11 days. (**C**) mCLEC-2 surface expression determined by flow cytometry following depletion by INU1 shows CLEC-2 was absent for at least 7 days after injection. Measurements with anti-rat and anti-mouse IgG excluded the possibility that the abolished anti-CLEC-2-FITC binding was due to the presence of remaining anti-CLEC-2 antibodies on the platelets (Suppl. Figure 8). (**D**) Depletion of CLEC-2 (using HEL1) had no effect on tail bleeding time. P >0.05, Fisher exact test. Horizontal lines represent the median time to cessation of bleeding with each circle representing one mouse. (**E**) Depletion of CLEC-2 had no effect on occlusive thrombus

◄ formation following mechanical injury of the abdominal aorta and hCLEC-2KI mice were comparable to WT. P >0.05, Fisher exact test. Horizontal lines represent the median time to vessel occlusion with each circle representing one mouse. For all experiments a minimum of 5 mice were tested per group. mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; MFI, mean fluorescent intensity.

(Figure 1B, Suppl. Figure 7E). Furthermore, HEL1 Fab fragments neither block rhodocytin induced platelet aggregation, unlike AYP1 Fab fragments,[16] nor AYP1 IgG induced aggregation of hCLEC-2^{KI} platelets (Suppl. Figure 7F). This not only shows that AYP1 and HEL1 act at different sites on CLEC-2 but also that CLEC-2 dimerisation at either site is sufficient to trigger platelet activation, as both antibodies, but not their Fab-fragments stimulate aggregation. To investigate whether human, like mouse, CLEC-2 can be immunodepleted *in vivo*, AYP1 or HEL1 were injected intraperitoneally and CLEC-2 surface expression was determined by flow cytometry. Both antibodies deplete CLEC-2 for at least 11 days with levels returning to normal by 18 days for AYP1 and 24 days for HEL1 (Figure 2B, Suppl. Figure 8). In both cases there was a transient thrombocytopenia lasting up to 4 days (Figure 2A). This is consistent with immuno-depletion of mCLEC-2 using INU1 however, the length of the CLEC-2 depletion is prolonged (Figure 2C).[8,17]

The effect of hCLEC-2 depletion was investigated in in vivo thrombosis and hemostasis models. Depletion had no effect on tail bleeding time (Figure 2D) which adds further support for CLEC-2 having a minor role in hemostasis.[13] In the mechanical injury of the abdominal aorta thrombosis model there was no difference in the time to vessel occlusion in CLEC-2 depleted hCLEC-2^{KI} mice compared to untreated controls and neither group differed significantly from WT mice (Figure 2E). Notably, in the ferric chloride-induced injury of the mesenteric arterioles hCLEC-2^{KI} mice displayed an increased time to occlusion with 9 out of 16 vessels failing to occlude. This was not the case for the hCLEC-2^{KI}-depleted mice, in which only 3 out of 14 vessels failed to

occlude (Suppl. Figure 9). Thus, hCLEC-2^{KI} mice apparently resemble CLEC-2 depleted *WT* mice, as mice lacking CLEC-2 were protected in this model of arterial thrombosis[8,13]. These results are in line with previous studies suggesting that the contribution of CLEC-2 to thrombosis differs depending on the type of injury or the vascular bed.[8,13,14] In addition, our data suggest that there is a difference in the roles of mouse and humanized CLEC-2 in arterial thrombosis with mouse CLEC-2 contributing to thrombus stability. We speculate that this discrepancy to previous reports on CLEC-2 deficient mice that displayed reduced thrombus formation[8,13,15,18] could be attributed to the postulated intravascular CLEC-2 ligand. This could explain the reduction in vessel occlusion in untreated hCLEC-2KI mice as the human receptor cannot interact with the mouse ligand to the same extent as the mouse receptor. In line with this hypothesis, it has recently been confirmed that mouse CLEC-2 contributes to arterial thrombosis and thrombus formation on collagen i*n vitro*, while competitive inhibition of human CLEC-2 had no effect on *in vitro* thrombus formation.[18] It is also possible that human CLEC-2 itself has, at least to some extent, an anti-thrombotic effect, which could explain the difference in vessel occlusion between hCLEC-2^{KI} and hCLEC-2^{KI}-depleted mice. Notably, hCLEC-2 can compensate for mCLEC-2 during development as well as in hemostasis suggesting a conserved interaction between CLEC-2 and podoplanin in hCLEC-2^{KI} mice. Here, we have demonstrated that hCLEC-2 can be immunodepleted and provide proof of principle that the hCLEC-2^{KI} mouse can be used to study test anti-hCLEC-2 agents *in vivo*.

Acknowledgments

This project has received funding from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No 766118. This work was supported by the CRC 1525 (project number 453989101) and

the TR240 (project number 374031971) of the Deutsche Forschungsgemeinschaft (DFG; German Research Foundation) and the Rudolf Virchow Center. We thank Stefanie Hartmann, Juliana Goldmann and Ewa Stepien-Bötsch for excellent technical assistance.

Conflict of interest

The authors declare no competing financial interests

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Supplemental Materials to Chapter 5

Methods

Blood donors

Blood samples were obtained from healthy volunteers after obtaining written informed consent in accordance with the Declaration of Helsinki and with approval from the Institutional Review Boards of the University of Würzburg.

Animals

Animal experiments were approved by the district government of Lower Franconia. Humanized CLEC2 (hCLEC-2^{KI}) mice were generated by replacing the mouse *Clec1b* gene on chromosome 6 with the corresponding region of the human *Clec1b* gene using CRISPR/Cas9. Mice were genotyped by PCR using the following primers: fwd: 5'-GCAAAACAAACCCCAAGTGTCCTGG-3', WT-rev: 5'-ATGCCCAAATTGCTGAAT GAGCCTT-3' and KI-rev: 5'-CCGTTATCCCCTTGACTTCTATGCCC-3' yielding a 247 bp PCR product for the WT allele and a 479 bp product for the KI allele.

Antibodies and reagents

The mouse anti-human CLEC-2 antibodies AYP1 and AYP2, and the rat-anti mouse CLEC-2 antibodies INU1 and INU2, as well as recombinant mouse podoplanin-FC, were generated in house as previously described,[1,2] whereas the AYP1-FITC conjugate was purchased from Biolegend. Anti-mouse and antirat IgG-FITC antibodies were from Dako and the GAPDH antibody from Sigma Aldrich. All other antibodies were from Emfret Analytics. ADP, apyrase, prostacyclin (PGI_2), fibrinogen and hematoxylin were from Sigma Aldrich. Horm-type collagen was from Nycomed Pharma, thrombin

from Roche and U46619 from Alexis Biochemicals; heparin was from Ratiopharm, eosin was from Roth. Protein G Sepharose was from GE Healthcare. Rhodocytin and collagen related peptide (CRP) were produced, as described previously.[3,4]

Mouse platelet preparation

Mice were bled from the retro-orbital plexus into heparin using a heparinized glass capillary. Blood was centrifuged twice at 300 g for 6 min to obtain the platelet-rich plasma (PRP) which was transferred into additional heparin prior to the second centrifugation. 0.02 µg/ml apyrase and 0.1 µg/ml prostacyclin were added to the PRP, which was further centrifuged at 800 g for 5 min to pellet the platelets. Cells were resuspended in Tyrode's buffer with apyrase and prostacyclin, counted and centrifuged again before being resuspended at 5 x 10^5 platelets/µl for aggregation or 3 x 10^5 platelets/µl for spreading assays and left to rest at 37°C for 30 min prior to use.

Human platelet preparation

Citrated blood was taken from healthy volunteers and further anticoagulated with acid citrate dextrose (ACD). PRP was obtained by centrifugation at 300 g for 20 min and 0.02 µg/ml apyrase, 0.1 µg/ml prostacyclin and 100 µl/ml ACD were added before centrifugation at 500 g for 10 min. The resulting platelets were resuspended in 2 ml Tyrode's buffer supplemented with apyrase, prostacyclin and 150 µl/ml ACD and further centrifuged at 500 g for 10 min. Platelets were counted and resuspended in Tyrode's buffer at 5 x 10^5 platelets/µl and left to rest at 37°C for 30 min prior to use.

Platelet aggregation

Washed platelets were further diluted with Tyrode's buffer supplemented with 2 mM $CaCl_2$ and 100 µg/ml human fibrinogen for all agonists except thrombin where Tyrode's

buffer without fibrinogen was used. Platelet aggregation was measured using a 4-channel aggregometer (APACT) under stirring conditions for 10 min after the addition of 5 µg/ml collagen, 0.01 U/ml thrombin, 0.24 µg/ml rhodocytin, 1-20 µg/ml HEL1 or 10 µg/ml of either INU1 or AYP1 antibodies or HEL1 Fab fragments. For aggregation using ADP PRP was obtained as described above but without the addition of extra heparin. It was diluted in Tyrode's buffer supplemented with 2 mM $CaCl_2$ and aggregation was measured for 10 min after the addition of 5 µM ADP.

Platelet spreading

Platelets were allowed to spread at 37°C for 30 min on fibrinogen and CRP or 60 min on podoplanin before being fixed. For spreading on fibrinogen 0.01 U/ml thrombin were added to the platelets immediately prior to spreading. Platelets were imaged using a Zeiss Axiovert 200 microscope in DIC mode fitted with a 100x objective. 5 fields of view per coverslip were imaged and the platelets were categorized into 4 groups: 1) un-spread platelets, 2) platelets forming filopodia, 3) platelets with lamellipodia and 4) fully spread platelets.

Western blotting

Mouse and human platelets were prepared as described above and resuspended at 1 x 10^6 platelets/µl in lysis buffer prior to centrifugation at 20,000 g for 10 min at 4°C to remove cell membranes. Lysates were mixed with reducing (AYP2) or non-reducing (AYP1, INU2 and HEL1) Laemmli buffer. Samples were separated by SDS-PAGE and transferred onto PVDF membranes which were blocked and then incubated with primary antibody. Membranes were incubated with secondary HRP-conjugated antibodies which were detected using ECL and an Amersham Imager.

Platelet count

Blood was collected in EDTA coated tubes and platelet count was measured using a ScilVet analyzer.

Flow adhesion assay

Coverslips were coated with 200 µg/ml Horm collagen overnight at 37°C then blocked in 1% BSA. Blood was diluted 1:2 in Tyrode's buffer supplemented with 2 mM $CaCl_2$ and platelets were labeled with a Dylight 488-conjugated anti-GPIX antibody. Blood was perfused over the coverslips at a shear rate of 1200 s^{-1} for 4 min and washed for a further 4 min with Tyrode's buffer supplemented with $CaCl_2$. Eight representative fields of view were imaged in both brightfield and fluorescence using a Leica DMI6000B microscope with a 63x objective. Images were then analyzed in terms of platelet surface coverage and volume (fluorescence integrated density) using Fiji.[5]

Flow cytometry

Glycoprotein expression. For determination of glycoprotein expression patterns, 50 µl of mouse blood was collected in heparin, further diluted 1:2 in PBS and incubated with saturating amounts of anti-GPIb, GPVI, GPV, GPIbβ, GPIX, αIIbβ3, β3, α2, α5, mCLEC-2 or hCLEC-2 FITC-conjugated antibodies. 500 µl of PBS were added prior to measuring surface expression on a BD FACS Celesta. For assessing CLEC-2 expression on human platelets whole blood was diluted 1:20 in PBS and incubated with 2 µl AYP1-FITC.

Platelet count. Blood was incubated with saturating amounts of anti-GPIX-FITC and anti-αIIbβ3-PE conjugated antibodies and double positive platelets were counted for 30 s.

Platelet activation. Blood was diluted with 1 ml of Tyrode's buffer and centrifuged twice at 800 g for 5 min, then resuspended in 750 µl of Tyrode's supplemented with 2 mM $CaCl_2$. Saturating amounts of JON/A-PE and anti-P-selectin-FITC antibodies were added as well as 10 µM ADP, 3 µM U46619, 0.001–0.1 U/ml thrombin, 0.1–10 µg/ml CRP, 1.2 µg/ml rhodocytin or 10 µg/ml AYP1 and incubated for 15 min. 500 µl PBS were added and platelet activation measured.

CLEC-2 depletion

Amounts of 3 µg/g bodyweight of either AYP1, HEL1 or INU1 were injected intraperitoneally and platelet count and CLEC-2 surface expression were measured for up to 25 days. Antibody binding to platelets was determined using anti-mouse (AYP1) or anti-rat (HEL1 and INU1) IgG-FITC antibodies; prior to incubation, blood was further diluted in 1 ml PBS and centrifuged at 800 g for 5 min to remove any unbound CLEC-2 antibody. Mice were depleted 5 to 10 days before *in vivo* experiments.

Tail bleeding time

Mice were anesthetized by intraperitoneal injection of medetomidine (0.5 µg/g; Pfizer, Karlsruhe, Germany), midazolam (5 µg/g; Roche, Grenzach-Wyhlen, Germany) and fentanyl (0.05 µg/g; Janssen–Cilag, Neuss, Germany). 1.5 mm of the tail was cut with a scalpel and blood was collected every 20 s using filter paper without touching the wound. This was done until bleeding ceased up to a maximum of 20 min.

Mechanical injury of abdominal aorta

The 6- to 12-week-old mice were anesthetized as described above and the abdominal aorta was exposed. A Doppler ultrasonic flow probe (Transonic Systems) was placed around the aorta and thrombosis was induced upstream by mechanical injury resulting

from a 15 s compression using forceps. Blood flow was monitored until either it ceased (complete vessel occlusion for 1 min) or 30 min had elapsed.

$FeCl_3$-induced occlusive thrombus formation in mesenteric arterioles

3- to 4-week-old mice were anesthetized as described above. After a midline abdominal incision was made, the mesentery was exteriorized and 35 to 60 μm-diameter arterioles were visualized at 10x magnification with an inverted microscope (Axiovert 200, Carl Zeiss) equipped with a 100-W mercury lamp (HBO) and a CoolSNAP-EZ camera (Visitron Systems). Endothelial damage was induced by application of a 3-mm^2 filter paper saturated with 20% $FeCl_3$. Metavue software was used to record adhesion and thrombus formation of fluorescently labeled platelets (DyLight488-conjugated anti-GPIX Ig derivative) for 40 min or until complete occlusion of the vessel (blood flow stopped for >1 min).

Histology

Intestine, brain, liver, spleen, lymph nodes and femora were removed and fixed before being dehydrated and embedded in paraffin. Sections were stained with hematoxylin and eosin and imaged using a Leica DMI400B microscope. Megakaryocytes were counted in spleen and femoral sections across 8 fields of view.

Antibody generation

CLEC-2 was immunoprecipitated from human platelet lysates using Protein G Sepharose beads coupled to AYP1 and used to repeatedly immunize female Wistar rats. Splenic B-cells were fused with Ag14 myeloma cells and HAT medium was used to select for hybridomas. These were tested by flow cytometry for secretion of hCLEC-2 specific antibodies; supernatant from each hybridoma was incubated with hCLEC-2^{KI}

mouse blood before washing in 1 ml PBS by centrifugation. The supernatant was discarded and the blood was incubated with anti-rat IgG-FITC before being analyzed on a BD FACS Calibur. Positive hybridomas were subcloned and tested for hCLEC-2 specificity twice before monoclonal antibody purification.

Statistical analysis

Normally distributed data, as determined using a Shapiro-Wilk test, are shown as mean ± standard deviation and were analyzed by ANOVA followed by multiple comparison correction where appropriate, unless otherwise stated. Data that do not follow a normal distribution are shown as median with interquartile range and were analyzed by Mann-Whitney tests unless otherwise stated.

Suppl. Table 1. Analysis of genotypes at weaning of pups from heterozygous hCLEC-2^{KI} matings.

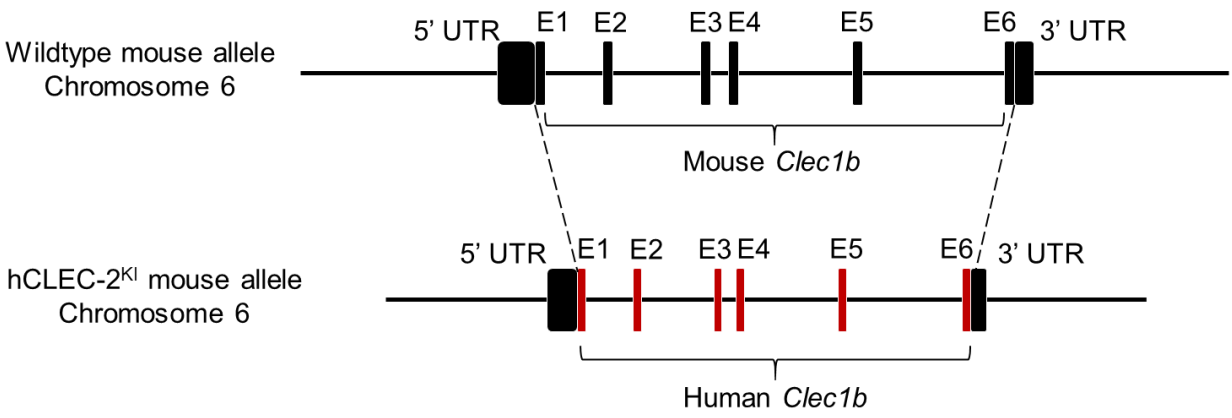
	Expected	**Observed**
Wildtype	52	63
Heterozygous	102	92
hCLEC-2^{KI}	52	51
Total	206	206
Chi^2		1.58 (P = 0.45)

Suppl. Table 2. Analysis of blood parameters shows no difference between wildtype and hCLEC-2^{KI} mice. Data are shown as median with the interquartile range in brackets, n = 12. WBC, white blood cell; LYM, lymphocyte; MON, monocyte; GRA, granulocyte; EOS, eosinophil; RBC, red blood cell; PLT, platelet; MPV, mean platelet volume; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width.

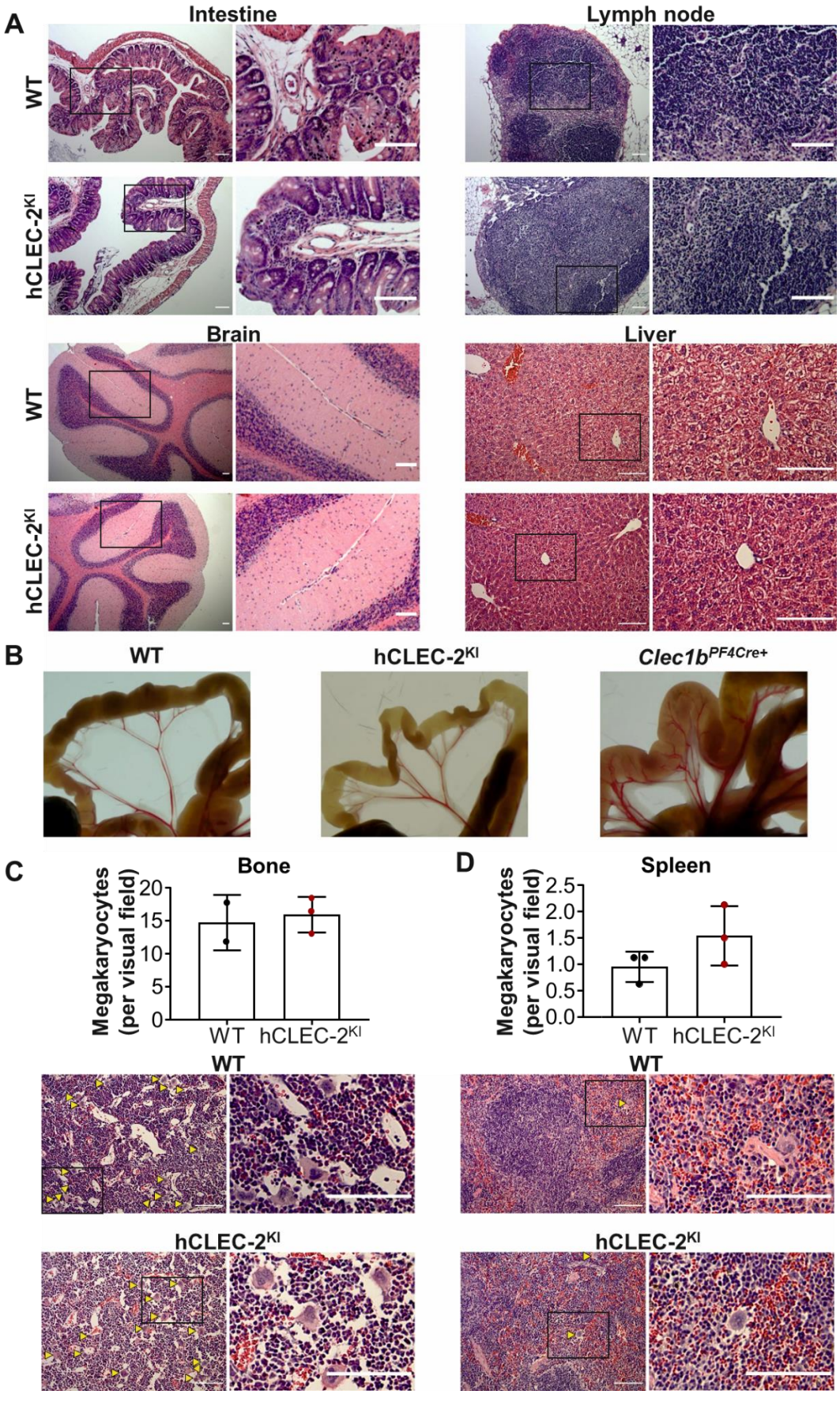
	WT	**hCLEC-2^{KI}**	**Mann-Whitney**
WBC 10^3/mm^3	4.45 (2.13)	4.25 (1.25)	P > 0.05
LYM 10^3/mm^3	3.50 (1.58)	3.55 (0.83)	P > 0.05
MON 10^3/mm^3	0.10 (0.08)	0.10 (0)	P > 0.05
GRA 10^3/mm^3	0.70 (0.28)	0.60 (0.35)	P > 0.05
EOS 10^3/mm^3	0.03 (0.02)	0.05 (0.01)	P > 0.05
RBC 10^6/mm^3	9.91 (0.66)	9.73 (0.48)	P > 0.05
PLT 10^3/mm^3	1056 (211)	1010 (278)	P > 0.05
MPV µm^3	5.55 (0.3)	5.65 (0.10)	P > 0.05
HGB g/dl	14.60 (0.95)	14.80 (0.75)	P > 0.05
HCT %	47.10 (3.23)	47.10 (2.10)	P > 0.05
MCV µm^3	48.00 (2.75)	48.50 (2.75)	P > 0.05
MCH pg	14.75 (0.75)	15.25 (0.82)	P > 0.05
MCHC g/dl	30.95 (0.57)	31.10 (0.75)	P > 0.05
RDW %	15.10 (0.65)	15.25 (1)	P > 0.05

Suppl. Table 3. Comparison of platelet glycoprotein expression shows no difference between wildtype and hCLEC-2^{KI} mice. Data are shown as median with the interquartile range in brackets. N = 4 and data are representative of 3 independent experiments.

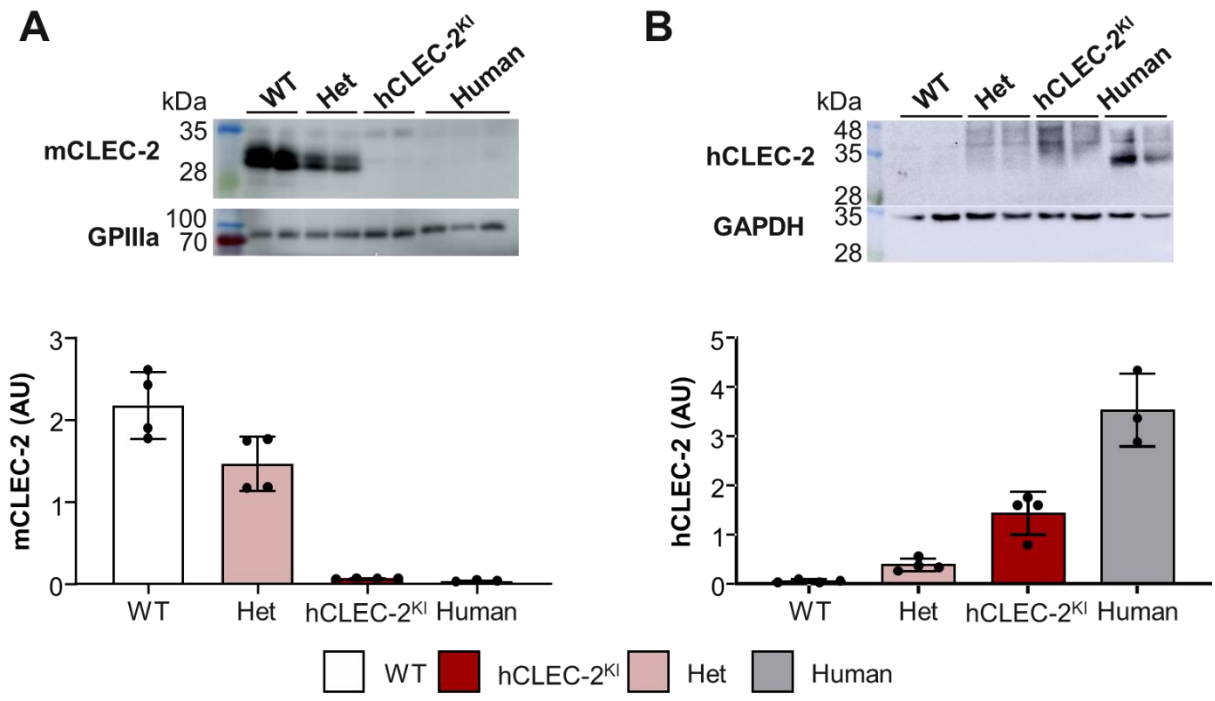
	WT	**hCLEC-2^{KI}**	**Mann-Whitney**
α-GPIbα	5608 (863)	5559 (288)	P > 0.05
α-GPVI	1702 (1453)	1959 (1901)	P > 0.05
α-GPV	4333 (232)	4165 (227)	P > 0.05
α-GPIbβ	7768 (764)	7922 (535)	P > 0.05
α-GPIX	8293 (385)	7997 (570)	P > 0.05
α-αIIbβ3	9341 (633)	9470 (740)	P > 0.05
α-β3	4647 (346)	4578 (878)	P > 0.05
α-α5	633 (104)	705 (92)	P > 0.05
α-α2	991 (118)	960 (129)	P > 0.05



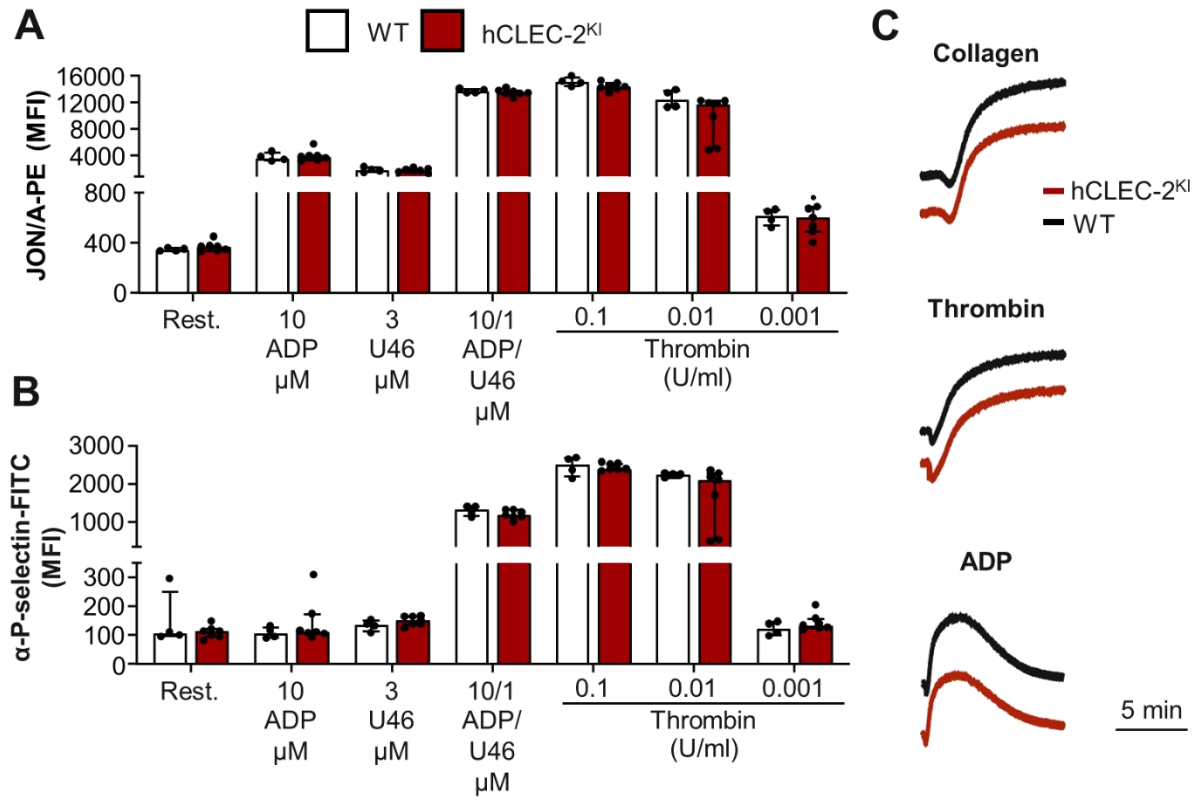
Suppl. Figure 1. Generation of hCLEC-2^{KI} mice. CRISPR/Cas9 was used to replace the wildtype mouse *Clec1b* gene on chromosome 6 with the human variant. The entire mouse gene was replaced with the human but the three and five prime untranslated regions (3' and 5' UTR) remained as the original mouse sequence.


A
Intestine
Lymph node
WT
hCLEC-2KI
Brain
Liver
WT
hCLEC-2KI
B
WT
hCLEC-2KI
Clec1bPF4Cre+
C
Bone
Megakaryocytes (per visual field)
20
15
10
5
0
WT
hCLEC-2KI
WT
hCLEC-2KI
D
Spleen
Megakaryocytes (per visual field)
2.5
2.0
1.5
1.0
0.5
0.0
WT
hCLEC-2KI
WT
hCLEC-2KI

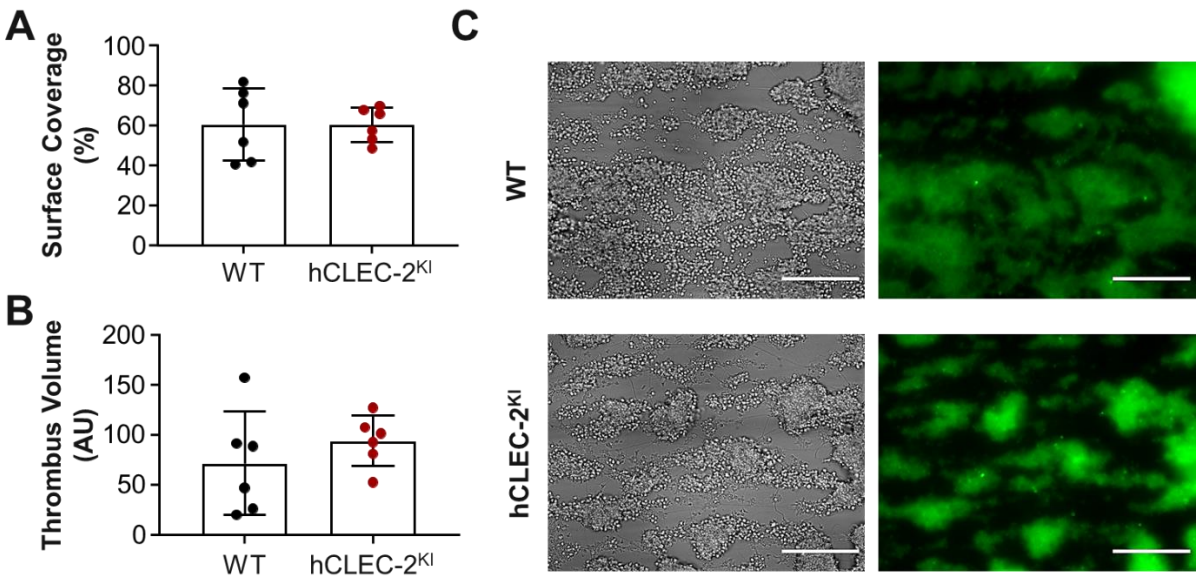
◄ **Suppl. Figure 2. No evidence of blood-lymphatic mixing and comparable organ morphology between hCLEC-2^{KI} and WT mice.** (**A**) Hematoxylin and eosin staining of intestines, lymph nodes, brains and livers from hCLEC-2KI and WT mice show no differences in morphology. (**B**) Intestines from adult WT, hCLEC-2KI and Clec1bPF4Cre^{+} mice were ◄compared for evidence of blood-lymphatic mixing. In Clec1bPF4Cre^{+} but not hCLEC-2KI mice there is increased blood filling in the mesenteric vessels. (**C**) Staining quantification of megakaryocytes in bone sections shows no difference between hCLEC-2KI and WT mice or in bone morphology. (**D**) Staining quantification of megakaryocytes in spleen sections show no difference between hCLEC-2KI and WT mice or in spleen morphology. Scale bars represent 100 µM. Yellow arrowheads indicate megakaryocytes. Images are representative of n = 3. Results are shown as means ± SD.



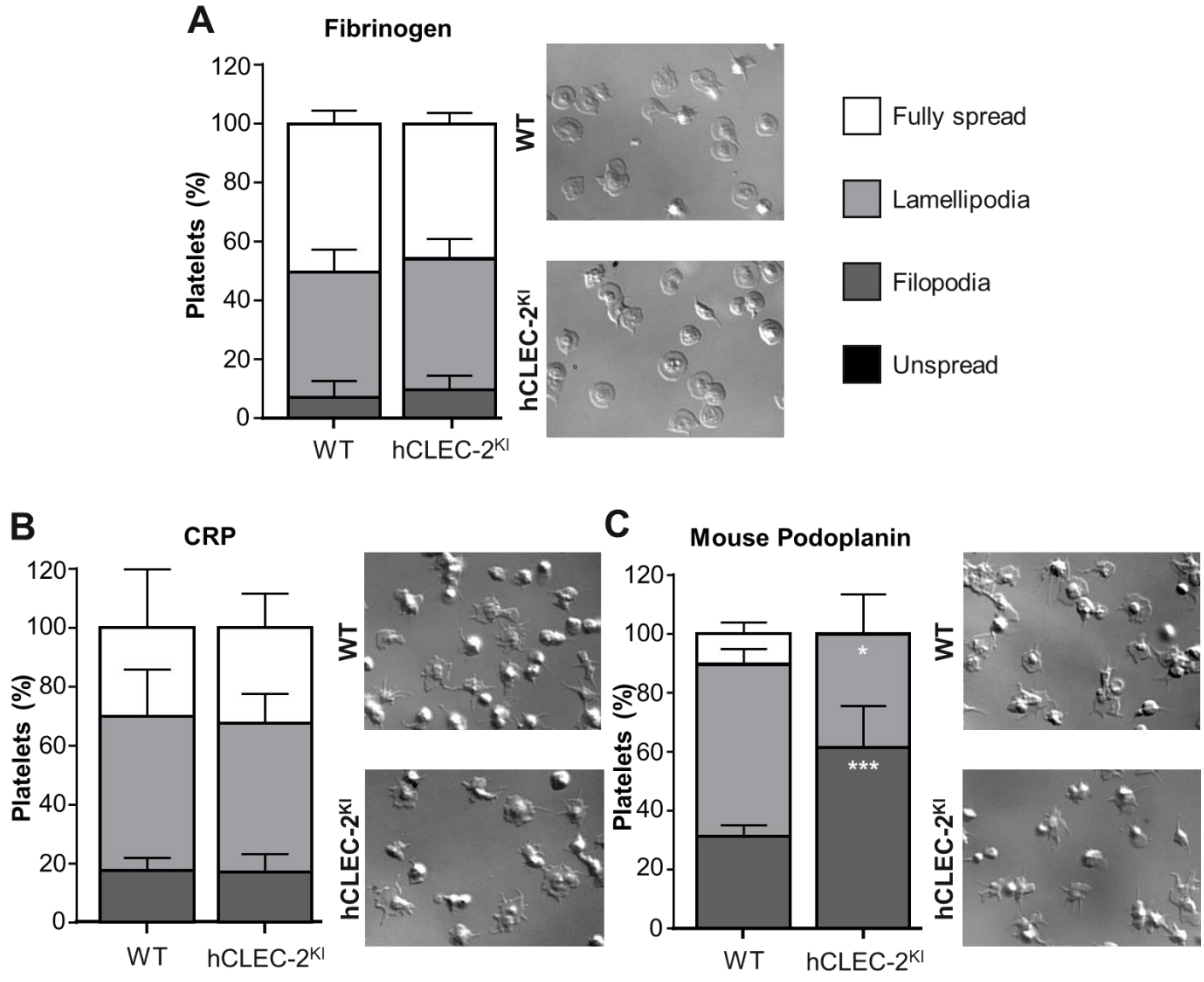
Suppl. Figure 3. hCLEC-2^{KI} mice only express human CLEC-2. Western blot analysis of platelet lysates from WT, heterozygous, hCLEC-2KI and healthy human donors show hCLEC-2KI mouse platelet express human but not mouse CLEC-2. CLEC-2 expression was normalized to the loading control GAPDH. (**A**) Representative Western blot and quantification of mouse CLEC-2 using the antibody INU2. (**B**) Representative Western blot and quantification of human CLEC-2 using the antibody AYP2. Results are shown as means ± SD, and each circle represents one individual. Het, heterozygous; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; AU, arbitrary units.



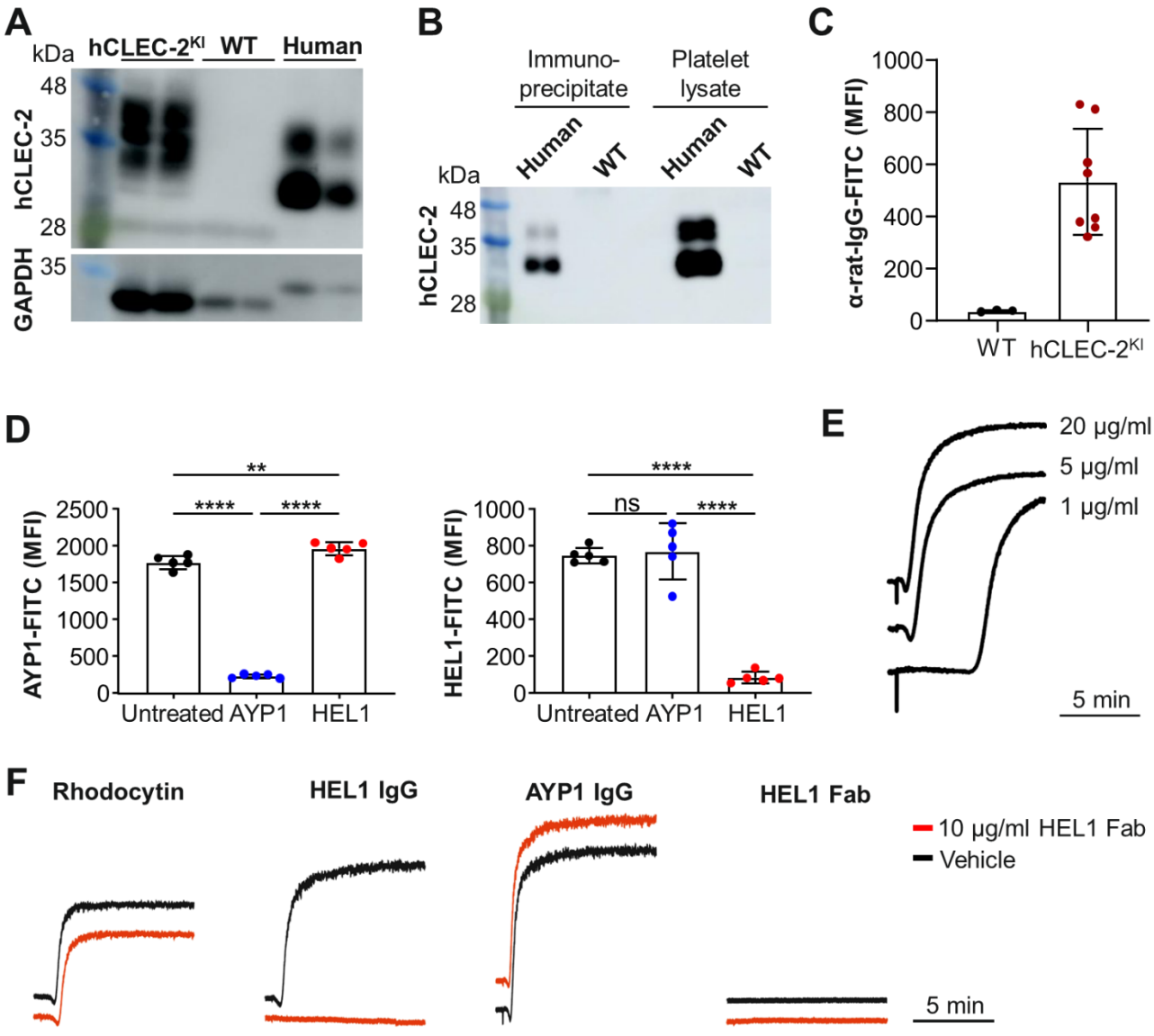
Suppl. Figure 4. hCLEC-2^{KI} mice have normal platelet activation and aggregation. (**A**) Platelet integrin activation measured by JON/A-PE binding in flow cytometry shows no difference in hCLEC-2KI compared to WT mice following stimulation with ADP, U46619 or thrombin. Data were analyzed using Mann-Whitney tests ($p > 0.05$) (**B**) Platelet granule secretion measured using an anti-P-selectin antibody in flow cytometry was unaltered in hCLEC-2KI following platelet activation by ADP, U46619 or thrombin. Data were analyzed using Mann-Whitney tests ($p > 0.05$) (**A**, **B**); each circle represents one individual. Results are shown as means ± SD. (**C**) Light transmission aggregometry with washed platelets shows unaltered aggregation in hCLEC-2KI mice in response to collagen (5 µg/ml) or thrombin (0.01 U/ml) as well as no difference in ADP (5 µM) induced aggregation in platelet rich plasma. Traces are representative of at least n = 3. *Abbreviations*: PE, phycoerythrin; MFI, mean fluorescent intensity; Rest, resting; ADP, adenosine diphosphate; U46, thromboxane A_2 mimetic U46619; FITC, fluorescein isothiocyanate.



Suppl. Figure 5. Thrombus formation on collagen under flow is unaltered in hCLEC-2KI mice at a shear rate of 1200 s^{-1}. (**A**) Surface coverage of thrombi formed on collagen was comparable between *WT* and hCLEC-2KI mice (unpaired t-test, p = 0.99). (**B**) Thrombus volume did not differ between *WT* and hCLEC-2KI mice (unpaired t-test, p = 0.36). (**C**) Representative brightfield and fluorescent images of aggregates formed under flow on collagen from WT and hCLEC-2KI mice. Platelets were labeled prior to flow with an anti-GPIX-Dylight-488 conjugated antibody. Scale bars represent 50 µm. Data are shown as means ± SD and each circle represents one animal. AU, arbitrary units.



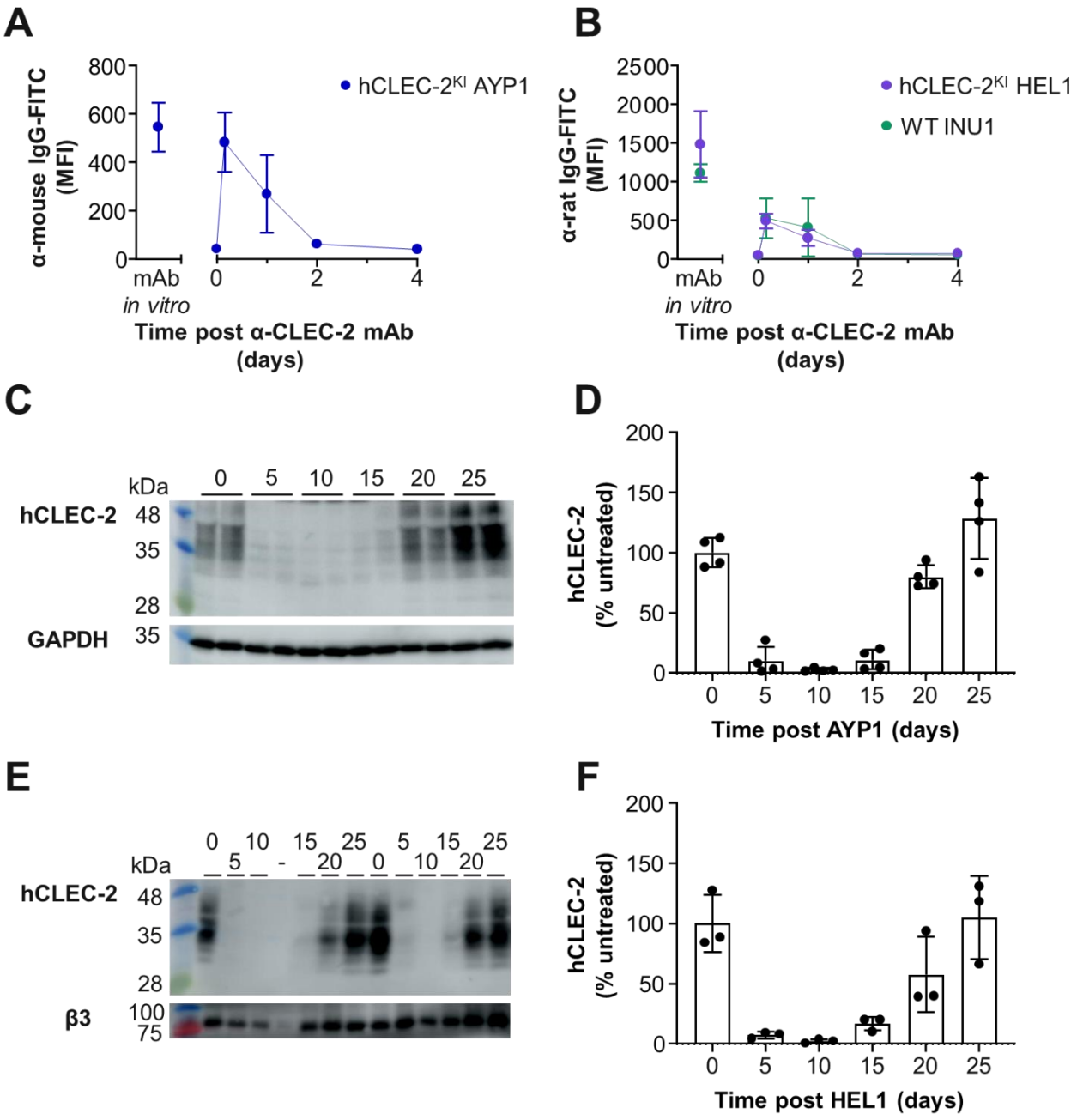
Suppl. Figure 6. Platelet spreading is unaltered on fibrinogen and CRP but reduced on mouse podoplanin in hCLEC-2^{KI} mice. Platelet spreading is shown as the percentage of platelets in each of the 4 stages of spreading: un-spread, forming filopodia, forming lamellipodia and fully spread platelets from 5 fields of view plus representative images. (**A)** Platelet spreading on 100 µg/ml human fibrinogen was comparable between WT and hCLEC-2KI mice after 30 min ($p = 0.63$). Thrombin 0.01 U/ml was added to the platelets immediately prior to spreading. (**B**) Platelet spreading on 10 µg/ml CRP after 30 min was comparable for WT and hCLEC-2KI mice ($p = 0.99$). (**C**) Platelet spreading on 10 µg/ml recombinant mouse podoplanin-FC was reduced in hCLEC-2KI mice compared to WT. More platelets had filopodia ($p = 0.0006$) in the hCLEC-2KI group and fewer formed lamellipodia ($p = 0.02$). However, no un-spread platelets were observed. Data were analyzed using a two-way ANOVA with Sidak's multiple comparison test. Data represent 3 mice per genotype, representative of at least 2 independent experiments. *** $p < 0.001$, * $p < 0.05$.



Suppl. Figure 7. HEL1 is specific to hCLEC-2 and appears to bind at a different site than AYP1 but still activates hCLEC-2^{KI} platelets. (**A**) HEL1 can detect hCLEC-2 from both hCLEC-2^{KI} and human platelet lysates, but not WT mouse lysates. The apparent difference in the molecular weight of hCLEC-2 from hCLEC-2^{KI} and human platelets is most likely the result of slightly different glycosylation patterns between both species. (**B**) HEL1 immunoprecipitates hCLEC-2. This is shown by Western blotting of the eluate following immunoprecipitation by HEL1 (5 µg) coupled to protein G sepharose beads from platelet lysate. (**C**) HEL1 can detect hCLEC-2 on the surface of hCLEC-2^{KI} but not WT platelets by flow cytometry. HEL was incubated with whole blood, which was then washed by centrifugation and an anti-rat IgG-FITC

◄ coupled antibody was used to detect HEL1 on the platelet surface. (**D**) HEL1 and AYP1 bind to different sites on hCLEC-2 as shown by flow cytometry. hCLEC2^{KI} mouse blood incubated with 10 µg/ml AYP1 had normal surface expression of hCLEC-2 determined using HEL1-FITC and blood incubated with 10 µg/ml HEL1 had normal hCLEC-2 surface expression when measured with AYP1-FITC. In both cases incubating and measuring hCLEC-2 surface expression with the same antibody resulted in a reduction in expression compared to an untreated control due to opsonization of the respective binding site. (**E**) HEL1 IgG causes dose-dependent aggregation of hCLEC-2KI platelets. (**F**) HEL1 Fab fragments do not block rhodocytin (0.24 µg/ml) or AYP1 IgG (10 µg/ml) induced hCLEC-2^{KI} platelet aggregation but do prevent HEL1 IgG (10 µg/ml) induced aggregation providing further evidence HEL1 and AYP1 bind to different epitopes on hCLEC-2. HEL1 Fab fragments themselves do not induce platelet aggregation. Data are show as means ± SD, and statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparisons test, ****p <0.001, **p <0.01; each circle represents one individual. *Abbreviations:* MFI, mean fluorescent intensity; FITC, fluorescein isothiocyanate.

►**Suppl. Figure 8. hCLEC-2 immuno-depletion by HEL1 and AYP1 results in CLEC-2 deficient platelets.** (**A**) AYP1 can be detected on the surface of platelets after intraperitoneal injection using an anti-mouse IgG-FITC conjugated antibody for only 1 day following injection. This is shown as an increased in MFI following antibody injection compared to the baseline measurements. (**B**) Traces of HEL1 and INU1 can be detected on the surface of hCLEC-2KI and WT platelets respectively for only 1 day after intraperitoneal injection using an anti-rat IgG-FITC conjugated antibody. This is shown as an increased in MFI following antibody injection compared to the baseline measurements. (**C**) Intraperitoneal AYP1 (3 µg/g bodyweight) depletes both intra- and extracellular hCLEC-2. Western blotting of platelet lysates from AYP1 injected mice at the various time points using AYP2 shows that hCLEC-2 is absent from platelets between 5 and 15 days after injection, earlier time points could not be studied due to the thrombocytopenia caused by AYP1 injection. (**D**) Quantification of Western blots showing hCLEC-2 depletion following AYP1 injection. hCLEC-2 levels increase slightly at 15 days after injection and return fully between day 20 and 25. Data is shown as the percentage of hCLEC-2 compared to untreated mice and normalized to the loading control GAPDH, each dot represents one animal. (**E**) Intraperitoneal HEL1 (3 µg/g bodyweight) depletes both intra- and extracellular hCLEC-2. Western blotting of platelet lysates from HEL1 injected mice at the various time points using HEL1 shows that hCLEC-2 is absent from platelets between 5 and 15 days after injection, earlier time points could not be studied due to the thrombocytopenia caused by HEL1. "-" indicates an empty lane. (**F**) Quantification of Western blots showing hCLEC-2 depletion following HEL1 injection. hCLEC-2 levels increase slightly 15 days after injection and return fully between day 20 and 25. Data is shown as the percentage of hCLEC-2 compared to untreated mice and normalized to the loading control integrin β3, each dot represents one animal. *Abbreviations:* MFI, mean fluorescent intensity; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.


A
α-mouse IgG-FITC (MFI)
800
600
400
200
0
hCLEC-2KI AYP1
mAb
in vitro
0
2
4
Time post α-CLEC-2 mAb (days)
B
α-rat IgG-FITC (MFI)
2500
2000
1500
1000
500
0
hCLEC-2KI HEL1
WT INU1
mAb
in vitro
0
2
4
Time post α-CLEC-2 mAb (days)
C
kDa
0 5 10 15 20 25
hCLEC-2
48
35
28
GAPDH
35
D
hCLEC-2 (% untreated)
200
150
100
50
0
0 5 10 15 20 25
Time post AYP1 (days)
E
0 10 15 25 5 15 25
kDa
5 - 20 0 10 20
hCLEC-2
48
35
28
β3
100
75
F
hCLEC-2 (% untreated)
200
150
100
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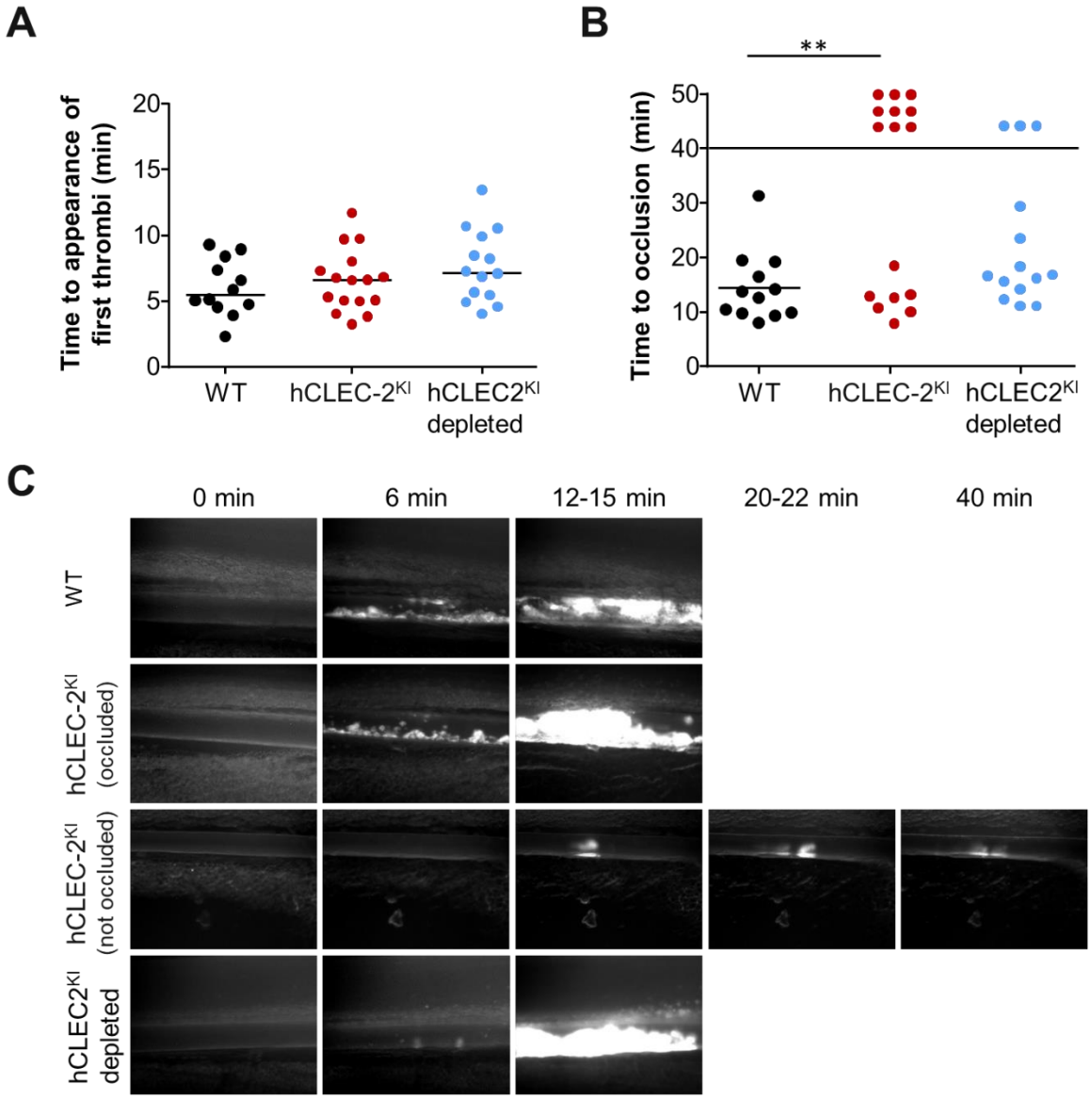
0
0 5 10 15 20 25
Time post HEL1 (days)



Suppl. Figure 9. Occlusive thrombus formation is reduced in hCLEC-2^{KI} mice following $FeCl_3$-induced injury. (**A**) No differences were observed in the time to appearance of the first thrombi following $FeCl_3$-induced injury in CLEC-2 depleted or naïve hCLEC-2 mice compared to *WT*. Data is shown as mean. (**B**) hCLEC-2^{KI} mice had increased vessel occlusion times compared to WT mice (Fisher exact test, p = 0.0028), while the differences to CLEC-2 depleted hCLEC-2^{KI} mice were not significant (Fisher exact test, p = 0.072). Horizontal lines represent the mean. (**C**) Representative images of mesenteric vessels following $FeCl_3$-induced injury for each group at the times indicated after injury. Representative images of both occluded and non-occluded hCLEC-2^{KI} vessels are shown. Each symbol represents 1 vessel and up to 3 vessels per mouse were injured; **p <0.01.

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Chapter 6

General discussion

1. Comprehensive overview of the thesis results

In this thesis, I describe several studies of novel strategies to target the platelet receptors GPVI and CLEC-2, and techniques to establish the intervention effects, in agreement with the aims of this thesis (chapter 1). Although mouse and human GPVI and CLEC-2 are similar in structure, the inter-species differences could still have led to a divergence in the roles of these receptors (Chapter 1). In case of GPVI, for instance human GPVI (huGPVI), but not mouse GPVI (mGPVI) bind fibrinogen to stimulate platelet spreading and activation.[1-4] The monoclonal antibody JAQ1 (rat IgG) is the first tool described to block the activity of mGPVI *in vitro* and to down-regulate the receptor *in vivo*; nowadays it is a widely used tool for platelet research.[5,6] The studies in this thesis have revealed that JAQ1 cross-reacts with huGPVI, but does not completely inhibit the GPVI-induced platelet activation. This indicates that, subtle differences between mGPVI and huGPVI influence ligand-receptor interaction and subsequent platelet activation. The finding that the effect of JAQ1 on huGPVI in platelets from the transgenic *hGP6*$^{tg/tg}$ mice is comparable to the effect of the antibody on human platelets underscores the suitability of using humanized mouse lines (Chapter 2). The transgenic mice thus allow an improved translational approach in the search for antagonists of candidate receptors for antiplatelet therapy.

The binding of platelet GPVI to collagen is considered to thrive thrombus formation in pathological conditions, for instance upon rupture of an atherosclerotic plaque, as this is enriched in collagen types I and III.[7,8] Herein, platelet GPVI acts in concert with other processes of coagulation and platelet activation. However, on which time scale the blocking of GPVI is effective has never been investigated. The results in Chapter 3 reveal that, under arterial shear conditions using human whole blood perfused over collagen and tissue factor, the activity of GPVI and of the downstream

tyrosine kinase Syk was confined to the first minutes of thrombus and fibrin formation. The same applied for tissue factor, as deduced from its blockage with inactivated factor VIIa. In other words, the inhibition of either GPVI or factor VIIa at a later time point than 2 min had no more than little effect on the thrombus buildup and fibrin deposition. In contrast, we found that the thrombin-dependent platelet activation via the G-protein coupled receptors PAR1 and PAR4 continued to be important also at later time points. The same was true for integrin αIIbβ3 activity in the formation of thrombi with multilayers of platelets.

In the last decades, plenty of studies have described GPVI as a candidate target for anti-platelet therapy,[6-11] which has led to the development of different compounds inhibiting GPVI-mediated platelet activation.[12-16] Recently, the first GPVI-directed signal-blocking drug, Glenzocimab, appeared to have positive results in a clinical trial,[17] which will boost the efforts to generate other GPVI inhibitors. In Chapter 4, I present an alternative approach, *i.e.* the scaled or complete depletion of huGPVI from the platelet surface as another lead for the development of anti-thrombotic drugs. The complete ablation of huGPVI from the platelet surface profoundly protected mice from arterial thrombotic events, without severely increasing the tail bleeding time. This strategy came with an unprecedented efficacy, half-life and cost-effectiveness and confirm the hypothesis of this thesis with regard to GPVI (Chapter 1).

As already extensively introduced (Chapter 1.6), platelet CLEC-2 is best known as a podoplanin receptor, critically contributing to the blood-lymphatic vessel separation during development. Furthermore, as already discussed (Chapter 1.6) CLEC-2 has also been described to contribute to hemostasis, thrombosis, thrombo-inflammation and tumor metastasis, raising the interest in this receptor as a potential therapeutic target. Whilst a humanized GPVI mouse line was already available as a useful model for *in vivo* studying and targeting this receptor, this was not yet the case

for CLEC-2. In Chapter 5, we present a humanized CLEC-2 mouse line is presented, and illustrate its potential for the evaluation of therapeutics is illustrated, including CLEC-2-selective antibodies, again with expected low bleeding side effects. This confirms the hypothesis of this thesis with regard to CLEC-2 (Chapter 1).

2. Novel approaches for targeting GPVI

The rat antibody JAQ1 was the first tool described to block GPVI activity of mouse platelets *in vitro* and to deplete, in its IgG form, the receptor from the surface of circulating platelet *in vivo*.[6] Since the initial observation that the JAQ1-induced depletion of platelet GPVI protected mice from experimental arterial thrombosis without severely impairing hemostasis,[18] the interest in GPVI as a candidate anti-thrombotic target rapidly increased. This thesis (Chapter 4) reports the first attempt of targeting huGPVI using JAQ1, in order to assess its potential for the generation of new anti-platelet therapeutics. Interestingly, *in vitro* assays pointed to a paradoxical effect of JAQ1 on huGPVI. Thus, pre-incubation of human platelets with JAQ1 led to an enhancement of GPVI-dependent activation in response to collagen or convulxin, whereas activation in response to CRP was partially suppressed.

These results led to the hypothesis that JAQ1 binds to a structurally conserved epitope on GPVI that differs in its receptor-activating effect in mice and human. Nonetheless, the observed inhibition of CRP-induced platelet activation indicates that part of the JAQ1-binding (and likely GPO-interacting) domain is still partially preserved in huGPVI, even when overridden by a huGPVI activating effect. Interestingly, in transgenic mice JAQ1 binds to huGPVI *in vivo*, but differently than to mGPVI in *WT* mice.[6] Regarding the humanized mouse platelets, JAQ1 only partially depleted their GPVI receptors, thus generating low-density huGPVI (huGPVI^{LO}) platelets in the

circulation. It appeared that already the partial depletion of huGPVI from the platelet surface was sufficient to suppress the collagen-dependent aggregate formation.

A possible therapeutic approach, where platelet GPVI is partially down-regulated in a pharmacological manner has not been tested so far. From a clinical point of view, this approach could be as beneficial as compared to the complete loss of GPVI, since this might still prevent or reduce arterial thrombosis. In man, it has been seen that inter-individual differences in GPVI expression alter the collagen-dependent platelet responses, which might reduce the risk of thrombosis.[19]

Chapters 3 and 4 also describe a monoclonal, huGPVI-specific antibody, EMF-1 (provided by Emfret Analytics, Germany, on a collaborative basis) with GPVI-blocking effects. When applied as a Fab fragment, the antibody inhibits thrombus formation, abrogates GPVI-dependent phosphatidylserine exposure, and thereby reduces fibrin deposition under conditions of arterial shear stress and active coagulation. In Chapter 4, EMF-1 is used in its IgG form to study its suitability for a GPVI-depletion approach to downregulate this receptor *in vivo*. The results demonstrate that EMF-1 was able to completely deplete huGPVI from the surface of circulating platelets, and it thereby induced an unprecedented long *in vivo* half-life, when compared to other published huGPVI-targeting strategies.[11,13,15]

Up to date, only two GPVI-directed drugs have been tested in clinical trials. The first peptide, Revacept, was developed as a competitive inhibitor for the GPVI-binding site on collagen. Revacept is a soluble, dimeric form of the GPVI ectodomain linked to an FcR domain, that binds to immobilized collagen and competes for its binding site with GPVI of circulating platelets.[20] After positive results in a phase I clinical trial,[15] a phase II randomized clinical trial including patients with stable ischemic heart disease undergoing elective percutaneous coronary intervention (PCI), showed so far no benefit in reducing myocardial injury.[21] The drug ACT017 (Glenzocimab), a humanized

monovalent antibody directed against human GPVI, has also been tested in clinical trials. The phase I trial of this compound revealed a terminal half-life of 10.2 hours in healthy volunteers and an efficient reduction of platelet aggregation *ex vivo* for up to 8 hours.[13] So far positive results in a phase II clinical trial with patients suffering from acute ischemic stroke,[17] have sparked the interest for ACT017 and other direct GPVI inhibitors. Although the depletion of GPVI from platelets has been proposed as another GPVI-targeted strategy, this has not been tested in humans up to date. In support of previous reports,[6,22,23] the mouse data in Chapter 4 provide good indications that this approach might be safe, effective and long lasting. Single treatment of *hGP6*$^{tg/tg}$ mice pointed to a profound protection from thrombotic events for up to 12 days, thus with a half-life by far exceeding that reported for Revacept[15] and Glenzocimab.[24] Nontheless, the study reported here only presented pre-clinical data and no test in human has been performed so far. Although the use of a humanized mouse line allowed us to assess the pharmacokinetic of the compound and its effect on thrombosis and hemostasis, this might still not complitely resemble the effect of this therapeutic strategy in a clinical setting. Nevertheless, the results reported in this thesis illustrate that the different strategies to target or deplete human GPVI are important to pursue.

3. Success of therapeutic strategies directed at platelet GPVI

Alongside the development of GPVI inhibitors, a consideration to make is in which clinical set-up these drugs could be beneficial. The initial human studies conducted with Revacept and Glenzocimab point to a reduced *ex vivo* collagen-induced platelet activation and thrombus formation, so far without bleeding effects.[13,15] However, the two drugs have been tested in different clinical settings. On the one hand, Revacept was administered in patients undergoing PCI,[21] whereas Glenzocimab was tested in

patients suffering from acute ischemic stroke.[17] In addition, in either case the drugs were perfused as add-on to standard therapy, namely dual anti-platelet therapy for the PCI, and recombinant tissue-type plasminogen activator (r-tPA) for the acute ischemic stroke patients. While Glenzocimab directly inhibits GPVI, Revacept competes for its binding to collagen, thus indirectly blocking its function. In mice, it appeared that the first strategy is more successful.[25]

In Chapter 3, the results obtained with human blood incubated with the EMF-1 Fab fragment or a Syk inhibitor unveiled a function of GPVI especially in the early phase of thrombus formation, with a quick loss of effectivity after deposition of the first fibrin fibrils. This indicated a most profitable use of such drugs in the early prevention of thrombotic events. This has also been reported by another group, showing that GPVI dictates the fibrin production in the first platelet layer on collagen, whereas its role is overruled by the action of thrombin in the subsequent thrombus buildup.[26] Nevertheless, Chapter 4 also shows that EMF-1 IgG treatment highly protected mice from arterial thrombosis. The EMF-1 IgG-treated animals generated only small and instable thrombi at the site of injury, as appeared from analysis of the blood flow dynamics. This agrees with our flow chamber studies, using human blood and EMF-1 Fab, which also resulted in the presence of small-sized, fibrin-poor thrombi. Chapter 3 also confirms a major role of thrombin-induced PAR1/4 receptor signaling in the human thrombus buildup, although blockade of these receptors did not affect phosphatidylserine exposure and fibrin deposition. Even late receptor blockage was able to suppress the thrombus formation. Comparably to GPVI, PAR inhibitors have been proposed as candidate therapeutics to treat thrombotic events with limited effects on hemostasis.[27-31] Our data demonstrate distinct effects of antagonizing the GPVI- and PAR-dependent platelet activation, supporting the idea that a possible synergism, but not redundancy, might be achieved in intervention studies. When tested in patients

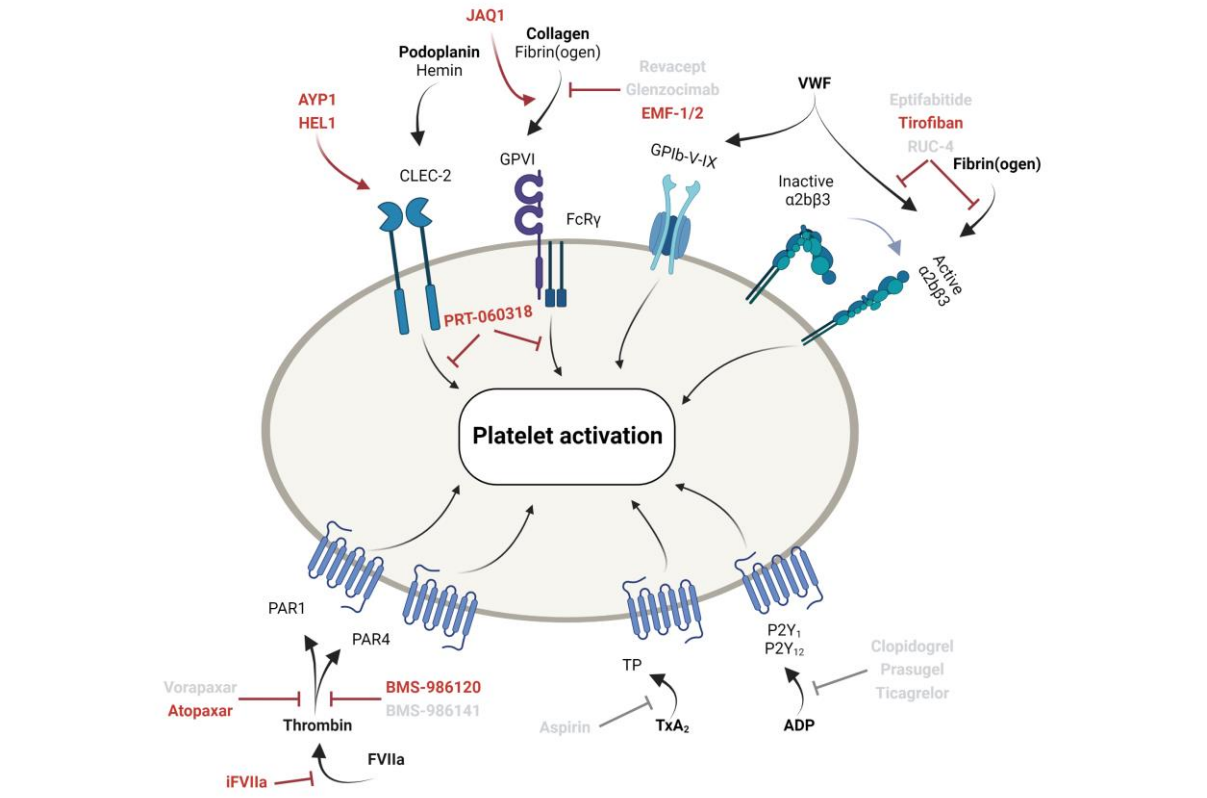

Figure 1. Overview of the platelet receptors and antagonists with a therapeutic effect or potential. In red are given the huGPVI/hCLEC-2-targeting molecules used in this thesis. In grey are inhibitors currently used for treatment or in clinical trials. Arrows indicate platelet activation via the concerning receptors, whereas blocking lines represent antagonizing actions.

with stable atherosclerosis, the PAR1 inhibitor vorapaxar, as add-on to standard therapy, reduced the risk of cardiovascular death or ischemic events, however at the cost of more frequent bleeding events.[32] Pre-clinical studies using guinea pig and cynomolgus monkeys demonstrated a low bleeding tendency after treatment with only the PAR4 inhibitor BMS-986120.[33,34] A first clinical trial involving healthy volunteers showed good tolerance in humans.[34] An overview of the platelet receptors and inhibitors used in this thesis is shown in Figure 1.

Previous reports showed that the combined genetic or pharmacological depletion of GPVI and CLEC-2 from mouse platelets resulted in a severe increase in bleeding tendency and abolishment of thrombus formation *in vivo*.[35] As GPVI and

CLEC-2 signal via essentially the same pathway, both receptors likely have partially redundant functions, explaining why the combined shut-down of two ITAM signaling receptors leads to a deleterious effect. On the other hand, a possible therapeutic strategy inhibiting GPVI plus one PAR receptor might prove to be more beneficial against thrombosis, while preserving the redundant signaling via CLEC-2 and the other PAR receptor. While it is known that PAR1 is responsive to lower thrombin levels than PAR4,[36] the two GPCRs indeed share the same G-proteins.[37]

Overall, the novel human GPVI-inhibiting EMF-1 Fab and the human GPVI-depleting full antibody EMF-1 may become useful for the generation of novel platelet-directed therapeutic agents and strategies to reduce cardiovascular disease targeting the GPVI receptor.

4. Using humanized mouse lines as valuable tools for translational studies

Humanized mice can overcome the obvious differences between human and mouse species, and can increase the translational relevance of biomedical studies.[38] Examples of humanized mouse models are rodents engrafted with human immune cells to study infection by Ebola virus,[39] or transgenic mice expressing human hepatocytes to better study hepatitis B and C.[40] Furthermore, a humanized model for the ACE2 receptor has been used to generate a mouse line susceptible to SARS-CoV-2 infection.[41]

In the thrombosis and hemostasis field, some humanized models have already successfully been generated. Mice expressing human factor XII were phenotypically indistinguishable from the *WT*, and allowed *in vivo* testing of human factor XII inhibitors.[42] The generation of a mouse line carrying human FcγRIIA-expressing platelets allowed to mimic human immune-mediated thrombocytopenic disorders.[43] A

study of full reconstitution of human platelets in mice indicated that macrophage depletion is necessary to boost the numbers of circulating platelets.[44] A humanized PAR4 mouse line highlighted differences between the human and mouse orthologs.[45] In Chapters 4 and 5, two humanized mouse models were employed to study GPVI and CLEC-2 receptor function and targeting. Figure 2 gives a schematic overview of the generation of the humanized mouse models used. A humanized mouse line for GPVI (*hGP6*$^{tg/tg}$) had already been generated by another group,[11] which allowed us to clarify the specific role of huGPVI in platelet activation upon fibrinogen binding.[4] Furthermore, recent evidence indicated that the role of the small GTPase-regulated protein Rac1 differs between human and mouse platelets in GPVI-dependent responses, as it was found to modulate the tyrosine phosphorylation of PLCγ2 in human nut not murine platelets.[46] Chapter 2 of this thesis shows that, although the anti-murine GPVI antibody JAQ1 was found to bind to huGPVI, it did not have the same inhibitory profile as on mGPVI. Capitalization on the *hGP6*$^{tg/tg}$ mouse model provided then novel insights into the pharmacokinetic of the new anti-human GPVI antibody EMF-1. When comparing in the living animals the effect of this compound to what is reported for the therapeutics currently undergoing clinical trials, (e.g. Glenzocimab), we found a major increase in the half-life, as well as a more profound protection in thrombosis models, whereas hemostasis was still unaltered. A longer half-life of an IgG compared to its Fab fragment in the circulation can explain the extended duration of this treatment. In addition, a complete absence of the receptor from the platelet surface might better protect from thrombosis respect to reversible inhibitors.

In addition, in Chapter 5 the work presents a novel humanized mouse line, in which the murine platelet CLEC-2 was replaced by the human orthologue. The normal viability of this mouse line indicated that the human CLEC-2 protein is able to reproduce the role of the murine receptor in embryonal development, such as in blood-lymphatic

vessel separation. It appeared that the humanized CLEC-2 platelets in mice expressed higher numbers of this receptor than seen in human platelets, but similar numbers of CLEC-2 as seen in WT mice. Also regarding their responsiveness to the agonist rhodocytin, the transgenic platelets phenocopied human platelets. In addition, this mouse line allowed *in vivo* targeting of human CLEC-2 with the novel antibody HEL1. The study demonstrated that pharmacological depletion of huCLEC-2 from platelets did not affect thrombotic or hemostatic responses.

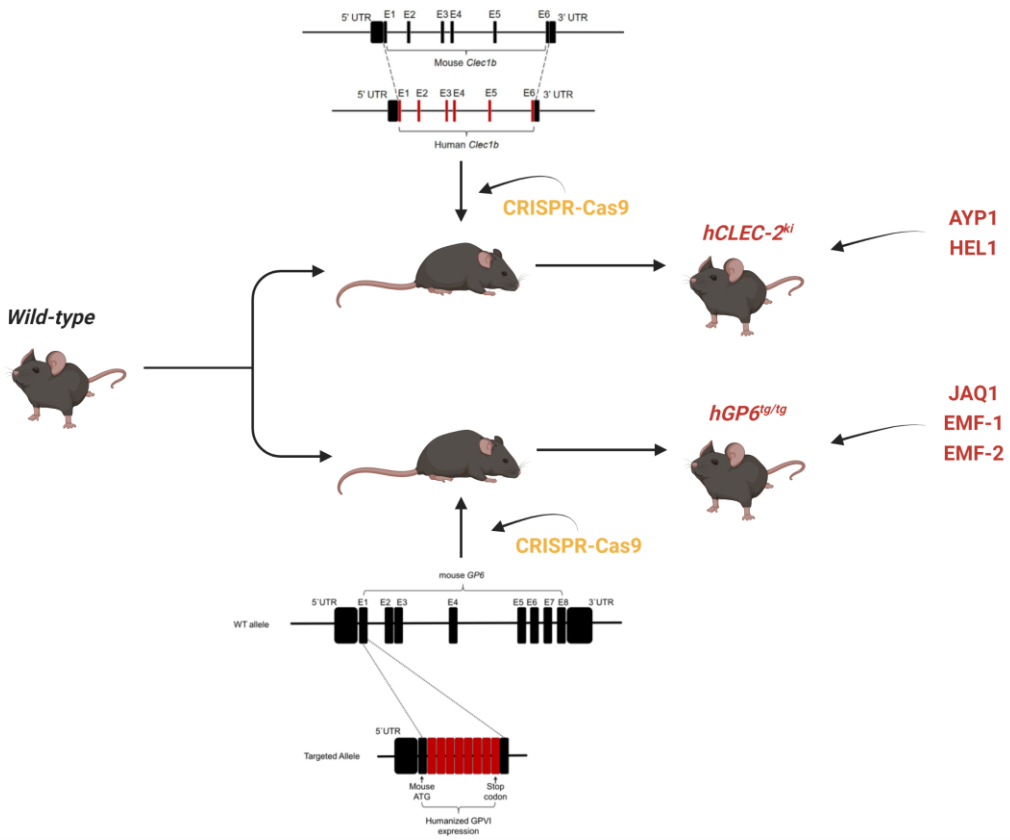

Figure 2. Overview of the generation of humanized GPVI (*hGP6*$^{tg/tg}$) and CLEC-2 (*hCLEC2*^{ki}) mouse lines. Wild type mice were genetically modified using CRISPR/Cas9 technology in order to silence expression of the target murine receptor and to replace it with the human ortholog *GP6* or *CLEC1b*. The generated mouse models were used to test different antibodies targeting the receptors, namely AYP1 and HEL1 for CLEC-2; and JAQ1 and EMF-1 for GPVI (in red). The CRISPR-Cas9 genetic engineering strategies are shown as miniature panel. The *hGP6*$^{tg/tg}$ generation strategy is described in Chapter 2. The hCLEC-2KI generation strategy is described in Chapter 5. Image created using Biorender.

5. Challenges and implications of platelet CLEC-2 targeting

Earlier mouse lines with deficiencies in CLEC-2 or podoplanin have provided insight into the role of this receptor. It appeared that a systemic defect in CLEC-2 (*Clec1b*$^{-/-}$)[47,48] resulted in neonatal lethality due to a dysregulation of the embryonic blood-lymphatic vascular separation.[49] On the other hand a defect in the CLEC-2 ligand podoplanin resulted in a 55% death in the first postnatal week, with only 20% mice surviving until adulthood.[49] Using platelet-specific, conditional *Clec1b* knockouts (*Clec1b*$^{fl/fl}$PF4-Cre), it was confirmed that CLEC-2 expression in the megakaryocyte-platelet lineage is required for normal development of the lymphatic and blood-brain vasculatures.[50] Chapter 5 presents the first generation of a mouse line expressing the human CLEC-2 form (hCLEC-2^{KI}). We could show that the hCLEC-2^{KI} mice are viable, fertile and without any sign of blood-lymphatic mixing, hence demonstrating functional redundancy of the mouse and human CLEC-2 proteins.

It was already known that CLEC-2 is expressed at a higher density on mouse platelets than on human platelets.[51,52] Interestingly, we find that the expression level density of hCLEC-2 on platelets is twice as high as in human platelets, reaching the level of *WT* CLEC-2 on platelets, thus pointing to a species-specific expression regulation. This finding is in contrast to previous proteomic estimates that CLEC-2 might have a 20-fold increased expression in mouse platelets.[52,53]

The platelet receptor CLEC-2 has been proposed as possible therapeutic target for several clinical conditions linked to its established roles. For instance, the CLEC-2-podoplanin axis is considered to regulate the severity of lung inflammation in mice.[55] In addition, an inflammatory role of CLEC-2 was assumed from the increased presence of CLEC-2–positive extracellular vesicles in plasmas from patients with rheumatoid arthritis.[54] Considering that certain tumor types over-express podoplanin,[56] which can interact with platelet CLEC-2, it is postulated that this event increases the survival of

tumor cells by physically protecting them from immune cells.[57] Also, a yet undefined role for CLEC-2 in hemostasis and thrombosis has been proposed.[58] Thus, it was shown that the combined depletion of CLEC-2 and GPVI more potently impaired these processes than the single depletion of either receptor.[35] The apparent redundancy of both receptors thereby points to common, signaling-dependent functions.[48,59]

The results of Chapter 5 now demonstrate that human and mouse CLEC-2 likely have different roles in thrombosis, with the mouse receptor more contributing to thrombus stability than human CLEC-2. However, inter-species differences (described in chapter 1.10) between CLEC-2 ligands may partly distort this picture. This might also explain why hCLEC-2 depletion did not affect thrombus formation following mechanical injury of the aorta. Given the results of Chapter 3 that human GPVI and Syk inhibition were similarly effective in suppressing whole-blood thrombus formation, there does not appear to be a major additional role of hCLEC-2 in this context. However, the natural CLEC-2 ligand in the healthy vasculature or plasma has not been described yet, thus leaving ample room for speculation. Knowing that podoplanin is no more than rarely present in atherosclerotic lesions, it has been hypothesized that smooth muscle cells might activate CLEC-2 via the calcium-binding protein, S100A13.[60,61] Another study provided indications that CLEC-2 deficiency affects deep venous thrombosis.[63]

Altogether, it appears that CLEC-2 is a relevant receptor with several pathophysiological roles. However, the targeting of CLEC-2 still faces challenges. For instance, CLEC-2 is not only expressed on platelets, but also on Kupffer cells and other immune cells.[58] Its known ligand podoplanin is expressed in multiple tissues, including brain, heart, kidney, lungs and lymphoid organs.[64] This abundant expression imposes the risk of side effects, when targeting the podoplanin-CLEC-2 interaction. So far, a molecule suitable for the *in vivo* targeting of human CLEC-2 has not been described. In mice, it was shown that CLEC-2 is downregulated with the antibody INU1.[59] In

Chapter 5, it is shown that the novel AYP-1 antibody can replicate this effect for hCLEC-2. In this respect, the inhibitory effect of katacine targeting human CLEC-2 could not be replicated in the mouse system, perhaps due to inter-species differences.[65] This also underlines the usefulness of the generated hCLEC-2^{KI} mice, as a valuable model to test the efficacy and safety of anti-hCLEC-2 molecules *in vivo*.

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Samenvatting

Medicatie gericht tegen bloedplaatjes speelt een belangrijke rol bij het verminderen of voorkomen van trombose, waardoor de levenskwaliteit en levensverwachting van patiënten met een hart- of herseninfarct toenemen. Momenteel is voor deze patiënten de gouden standaard het voorschrijven van aspirine en remmers van de $P2Y_{12}$-receptor, die erop gericht zijn de processen van plaatjesactivering te blokkeren middels respectievelijk tromboxaan A_2 en ADP, welke beide belangrijk zijn voor de plaatjesactivering. Deze duale antiplaatjes-therapie veroorzaakt echter ook een verhoogd risico op bloedingen. In dit proefschrift is mijn hypothese dat de gerichte onderdrukking van de ligand-geïnduceerde clustering van de collageenreceptor glycoproteïne VI (GPVI) of de podoplanine-receptor (C-type lectin-like receptor 2, CLEC-2) in plaatjes resulteert in een antitrombotisch effect met minimale bijwerking op de normale hemostase. Om dit na te gaan, heb ik de trombose- en hemostaseprofielen bepaald van muizen met een CRISPR-Cas9-genmodificatie resulterend in plaatjes met humane GPVI- of CLEC-2-receptoren. Voor deze karakterisering heb ik gebruik gemaakt van zowel *in vitro* als *in vivo* methoden. Daarnaast heb ik de muismodellen gebruikt om nieuwe moleculaire remmers te valideren, die gericht zijn tegen het humane GPVI of CLEC-2.

Hoofdstuk 1 geeft een algemene inleiding op het werk dat in dit proefschrift wordt gepresenteerd, met de nadruk op de functies van de verschillende receptoren bij bloedplaatjesadhesie, -aggregatie en trombus vorming in gezondheid en ziekte. Daarnaast geeft dit hoofdstuk een introductie in de huidige behandelingen met plaatjesremmers, en vat het recente vorderingen samen van behandeling gericht op het blokkeren van GPVI en CLEC-2.

In **hoofdstuk 2** laat ik voor het eerst zien dat het antilichaam JAQ1, gericht tegen muis GPVI, ook reageert met humaan GPVI (hGPVI), maar niet met GPVI van andere zoogdiersoorten, zoals ratten, konijnen, cavia's, varkens en honden. Ik laat verder zien

dat JAQ1 de functies van GPVI op de bloedplaatjes van muizen en mensen ten dele verschillend beïnvloedt. Net als bij muizenplaatjes, remt JAQ1 de activering van humane plaatjes, wanneer deze wordt geïnduceerd door collageen-gerelateerd peptide (CRP). In tegenstelling tot muizenplaatjes, remt JAQ1 in humane plaatjes de collageen-geïnduceerde adhesie, activering en aggregatie niet, maar veroorzaakt het in plaats daarvan een verhoogde respons. Verder geven mijn resultaten aan dat dit versterkende effect ook aanwezig is in de plaatjes van humane GPVI knock-in ($hGP^{6tg/tg}$) muizen.

In **hoofdstuk 3** heb ik de tijdsafhankelijke component bestudeerd van verschillende mechanismen van de humane plaatjes- en stollingsactivering, respectievelijk getriggerd door GPVI en tissue factor (TF), die een rol spelen bij de vorming van een bloedstolsels. Voor dit doel heb ik de volbloedtest met de Maastricht flow kamer zodanig aangepast, dat op gewenste tijdstippen een acute farmacologische interventie kon worden uitgevoerd. Met behulp van deze methode heb ik een tijdsafhankelijke, maar toch cruciale rol van GPVI en het Syk-kinase eiwit kunnen aantonen, die evenals de TF/factor VIIa-geïnduceerde coagulatie beperkt was tot de eerste twee minuten van de trombusopbouw. Om GPVI te blokkeren, heb ik in deze assay gebruik gemaakt van een nieuwe anti-GPVI Fab, namelijk EMF-1. Aan de andere kant bleek dat de plaatjesactivering via de trombine receptoren (protease-activated receptoren (PAR) 1 en 4) en het integrine αIIbβ3 meer langdurig actief was, en nog doorging tijdens latere stadia van de trombusvorming. Ten slotte heeft dit werk een prominente rol aangetoond van de GPVI- en Syk-afhankelijke signaleringsroute bij het genereren van zogenaamde procoagulante bloedplaatjes, middels expositie van het negatief geladen fosfolipide fostatidylserine.

Hoofdstuk 4 omvat een uitgebreide studie naar de depletie van humaan GPVI (huGPVI) in muizen met behulp van het transgene $hGP6^{tg/tg}$ model. Ten eerste laat ik zien dat behandeling van de muizen met het monoklonale antilichaam JAQ1 leidt tot een

gedeeltelijke depletie van GPVI *in vivo*, waardoor bloedplaatjes met een lage GPVI-dichtheid ($GPVI^{LO}$) worden gegenereerd, welke niet meer reageren op de liganden CRP of convulxine. *Ex vivo* flow kamer experimenten in volbloed wezen op een verminderd vermogen om plaatjesaggregaten op collageen te genereren bij arteriële bloedstroomsnelheden na behandeling van de muizen met JAQ1. Aanvullende studies met een ander anti-muis monoklonaal antilichaam tegen GPVI, JAQ4, in combinatie met wildtype muizen, toonden aan dat het genereren van het $GPVI^{LO}$-fenotype afhankelijk is van antilichamen met een lage affiniteit. Verder heb ik in hetzelfde hoofdstuk aangetoond dat de specifieke en hoge affiniteit anti-huGPVI antilichamen EMF-1 en EMF-2 in staat zijn om de receptor in $hGP6^{tg/tg}$ muizen *in vivo* volledig te downreguleren. In verschillende experimentele modellen bleken de met EMF-1 IgG behandelde muizen goed beschermd te zijn tegen occlusieve arteriële trombusvorming, terwijl staartbloedingstijden slechts minimaal werden beïnvloed. Met deze resultaten laat ik zien dat de *in vivo* downregulatie van huGPVI in potentie een veilige en effectieve strategie is om de activering van plaatjes te onderdrukken en om trombotische aandoeningen te behandelen.

Het werk in **hoofdstuk** 5 beschrijft de eerste karakterisering van een muizenlijn met een gehumaniseerde vorm van CLEC-2 ($hCLEC\text{-}2^{KI}$), en laat zien dat deze muizen kunnen helpen bij de evaluatie van nieuwe antiplaatjes-therapieën gericht tegen CLEC-2. Ten eerste laat ik zien dat de transgene muizen die alleen hCLEC-2 tot expressie brengen, niet te onderscheiden zijn van wildtype muizen (met muizen-CLEC-2-receptoren), met betrekking tot bloed-lymfevatscheiding, orgaan-ontwikkeling en morfologie. Ik heb ook aangetoond dat de bloedplaatjes van $hCLEC\text{-}2^{KI}$-muizen op een vergelijkbare manier als wildtype bloedplaatjes spreiden, activeren en aggregeren. Een belangrijke bevinding is, dat een nieuw anti-hCLEC-2-antilichaam, HEL-1, de hCLEC-2 receptoren *in vivo* blijkt te downreguleren; en bovendien dat een volledige downregulatie

geen effect heeft op de hemostase. Aan de andere kant laat ik zien dat de hCLEC-2KI-muizen een verminderde trombusvorming vertonen na $FeCl_3$ geïnduceerde beschadiging van de mesenteriale arteriolen, in tegenstelling tot in wildtype muizen, waarin volledige occlusie werd waargenomen in alle beschadigde arteriolen. Paradoxaal genoeg herstelt de hCLEC-2-downregulatie bij hCLEC-2KI-dieren het wildtype fenotype.

Ten slotte bediscussieer ik in **hoofdstuk 6** de resultaten van dit proefschrift en vergelijk ik deze kritisch met de huidige literatuur. In het bijzonder benadruk ik de relevantie en het belang van bloedplaatjesonderzoek met betrekking tot verschillende op GPVI gerichte strategieën, de mogelijkheden van het gebruik van gehumaniseerde muismodellen in translationeel onderzoek, evenals de implicaties en uitdagingen van toekomstige therapeutische strategieën gericht op CLEC-2.

Summary

Antiplatelet therapy plays an important role in reducing or preventing (the risk of) thrombotic events, thus increasing life quality and expectancy of patients with a heart or brain infarction. Nowadays, the gold standard for such patients is the use of aspirin and $P2Y_{12}$ receptor inhibitors, targeting platelet activation processes dependent on thromboxane A_2 and ADP, respectively, both of which are important second mediators of platelet activation. However, treatment with dual antiplatelet drugs coincides with an increased risk of bleeding events. In this thesis, my hypothesis is that the targeted modulation in platelets of ligand-induced clustering of the collagen receptor glycoprotein VI (GPVI) or the podoplanin receptor (C-type lectin-like receptor 2, CLEC-2) provides anti-thrombotic protection with minimal effect on normal hemostasis. To address this, I have characterized the thrombosis and hemostasis profiles of CRISPR-Cas9-modified mice with platelets expressing the human form of GPVI or CLEC-2 receptors, using both *in vitro* and *in vivo* methods. In addition, I have used these mouse models to validate novel inhibitors directed against human GPVI or CLEC-2.

Chapter 1 provides a general introduction to the work presented in this thesis, with emphasis on the functions of various receptors in platelet adhesion, aggregation and thrombus formation in health and disease. In addition, this chapter gives an introduction into the current standards of antiplatelet therapy, and it summarizes recent advances in the targeting of GPVI and CLEC-2.

In **chapter 2**, I show for the first time that the anti-mouse GPVI antibody JAQ1 does cross-react with human GPVI (huGPVI), but not with the GPVI orthologues of other tested mammalian species, such as rats, rabbits, guinea pigs, swine, and dogs. I further show that the JAQ1 antibody affects in part differently the functions of mouse and human platelet GPVI. Similarly, to mouse platelets, JAQ1 inhibits the activation process of human platelets, when induced by collagen-related peptide (CRP). However, unlike

mouse platelets, it does not inhibit the collagen-induced adhesion, activation and aggregate formation of human platelets, but it instead causes an increased response. Furthermore, my results indicate that this enhanced effect is also present in the platelets from human GPVI knock-in (*hGP6*$^{tg/tg}$) mice.

In **chapter 3**, I have studied the temporal roles of different pathways of human platelet and coagulation activation, triggered by collagen and tissue factor (TF), respectively, in whole-blood thrombus formation. For this purpose, I adapted the microfluidics whole-blood assay using the Maastricht flow chamber to acutely intervene pharmacologically in the thrombus formation process at desired time points. With the help of this method, my experiments revealed time-restricted, yet crucial, roles of GPVI and Syk protein kinase as well as TF/factor VIIa-induced coagulation during the first two minutes of thrombus buildup. In order to block GPVI, I employed a novel anti-GPVI Fab, EMF-1. On the other hand, it appeared that the platelet activation processes through thrombin receptors (protease-activating receptors 1 and 4) and integrin αIIbβ3 were prolongedly active, and continued during later stages of thrombus formation. Finally, this work demonstrated a prominent role of the GPVI- and Syk-dependent signaling pathway in the generation of coagulation-active platelets, exposing the negatively charged phospholipid phosphatidylserine.

Chapter 4 is a comprehensive study on the depletion of human GPVI (huGPVI) in mice using transgenic *hGP6*$^{tg/tg}$ mice. First, I show that treatment of the mice with the monoclonal antibody JAQ1 leads to the partial depletion of GPVI *in vivo*, thereby generating low-density GPVI (GPVI^{LO}) platelets, which were no longer responsive to the ligands CRP or convulxin. Blood from mice treated with JAQ1 showed an impaired capacity to generate platelet aggregates on collagen at arterial shear stress conditions in whole-blood flow studies. Additional studies with another anti-mouse GPVI monoclonal antibody JAQ4, in combination with wild-type mice, proved that the

generation of the GPVI^{LO} platelet phenotype is dependent on low-affinity antibodies. Furthermore, in this chapter, I have demonstrated that the specific and high-affinity anti-huGPVI antibodies EMF-1 and EMF-2 are able to fully downregulate the receptor in *hGP6$^{tg/tg}$* mice *in vivo*. In different experimental models, the EMF-1 IgG-treated mice appeared to be profoundly protected against occlusive arterial thrombus formation, whereas tail bleeding times were only minimally affected. With these results, I show that *in vivo* down-regulation of huGPVI has the potential to become a safe and effective strategy to target platelet activation and to treat thrombotic conditions.

The work presented in **chapter 5** describes the first characterization of a mouse line humanized for CLEC-2 (hCLEC-2^{KI}), and illustrates that these mice can help in the evaluation of novel therapeutics targeting CLEC-2. First, I show that the transgenic mice only expressing hCLEC-2 are indistinguishable from wildtype mice (with mouse CLEC-2 receptors), in terms of blood-lymph vessel separation and organ development and morphology. I also prove that the platelets from hCLEC-2^{KI} mice become activated, aggregated and spread in a comparable way as wildtype platelets. An important finding is that the novel anti-hCLEC-2 antibody HEL-1 can completely downregulate hCLEC-2 *in vivo;* and furthermore, that this downregulation has no effect on hemostasis. On the other hand, I show that the hCLEC-2^{KI} mice present with an impaired thrombus formation after $FeCl_3$-induced injury of mesenterial arterioles, in contrast to wildtype mice where complete vessel occlusion was observed in all tested arterioles. Paradoxically, the hCLEC-2 depletion in hCLEC-2^{KI} animals reverts the phenotype to a wildtype-like phenotype.

Finally, in **chapter 6** I discuss the results of this thesis, and critically compare these to the current literature. In particular, I emphasize the relevance and importance of platelet research on different GPVI-targeting strategies, the possibilities of the use of

humanized mouse models in translational research, as well as the implications and challenges of future therapeutic strategies targeting CLEC-2.

Zusammenfassung

Die pharmakologische Hemmung der Thrombozytenaggregation ist eine bedeutende Behandlungsmethode zur Verringerung oder Verhinderung thrombotischer Ereignisse, die die Lebensqualität und Lebenserwartung von Patienten mit einem Herz- oder Hirninfarkt erhöht. Der aktuelle Goldstandard für Patienten ist die Verwendung von Aspirin und $P2Y_{12}$-Rezeptor-Inhibitoren, die die Thrombozytenaktivierung durch Thromboxan A2 bzw. ADP - zwei wichtige *second-wave* Mediatoren - hemmen. Die Behandlung mit diesen dualen Thrombozyten-aggregationshemmern geht jedoch mit einem erhöhten Risiko für Blutungsereignisse einher. Die meiner Arbeit zu Grunde liegende Hypothese ist, dass die gezielte Modulation der liganden-induzierten *Clusterbildung* des Kollagenrezeptors Glykoprotein VI (GPVI) oder des Podoplaninrezeptors (C-Typ-Lektin-ähnlicher Rezeptor 2, CLEC-2) in Blutplättchen einen vergleichbaren anti-thrombotischen Schutz bietet, jedoch nur eine geringe Auswirkung auf die Hämostase hat. Um dies zu untersuchen, habe ich die Thrombose- und Hämostaseprofile von CRISPR-Cas9-modifizierten Mäusen, deren Blutplättchen jeweils die humane Form von GPVI oder CLEC-2 exprimieren, mit *in vitro* und *in vivo* Methoden charakterisiert. Darüber hinaus habe ich diese Mausmodelle verwendet, um neuartige Inhibitoren zu validieren, die gegen humanes GPVI oder CLEC-2 gerichtet sind.

Kapitel 1 beinhaltet eine allgemeine Einführung in die Thematik dieser Dissertation. Der Schwerpunkt liegt auf der Beschreibung der Funktionen verschiedener Rezeptoren der Thrombozytenadhäsion, -aggregation und Thrombusbildung unter (patho-)physiologischen Bedingungen. Darüber hinaus gibt dieses Kapitel eine Einführung in die aktuellen Standards der Thrombozytenaggregationshemmung und fasst die jüngsten Fortschritte beim pharmakologischen Angreifen (*targeting)* von GPVI und CLEC-2 zusammen.

In **Kapitel 2** zeige ich erstmalig, dass der gegen murines GPVI gerichtete Antikörper JAQ1 mit humanem GPVI kreuzreagiert, aber nicht mit GPVI-Orthologen anderer getesteter Säugetierspezies (wie Ratte, Kaninchen, Meerschweinchen, Schwein und Hund). Ich zeige weiterhin, dass der JAQ1-Antikörper die Funktionen von murinen und menschlichem GPVI teils unterschiedlich beeinflusst. Ähnlich wie bei Mausthrombozyten hemmt JAQ1 den Aktivierungsprozess menschlicher Thrombozyten, wenn dieser durch ein synthetisches Collagenpeptid (*collagen-related peptide,* CRP) ausgelöst wird. Im Gegensatz zu Mausthrombozyten hemmt es jedoch nicht die Kollagen-induzierte Adhäsion, Aktivierung und Aggregatbildung menschlicher Blutplättchen, sondern bewirkt stattdessen eine verstärkte Antwort. Darüber hinaus deuten meine Ergebnisse darauf hin, dass diese verstärkende Wirkung auch in den Blutplättchen von humanen GPVI-Knock-in (*hGP6*$^{tg/tg}$)-Mäusen vorhanden ist.

In **Kapitel 3** habe ich die Zeitabhängigkeit verschiedener Signalwege der Thrombozyten- und Gerinnungsaktivierung bei der Bildung von Thromben - induziert durch Kollagen bzw. Gewebefaktor (*tissue* factor, TF) - im humanen Vollblut untersucht. Zu diesem Zweck habe ich den auf der Maastricht-Flusskammer basierenden Mikrofluidik-Vollbluttest angepasst, um zu gewünschten Zeitpunkten akut pharmakologisch in den Thrombusbildungsprozess einzugreifen. Mit Hilfe dieser Methode zeigten meine Experimente zeitlich begrenzte, aber entscheidende Rollen von GPVI und der Proteinkinase Syk sowie der von TF/Faktor VIIa-induzierten Gerinnung während der ersten zwei Minuten der Thrombusentstehung. Um GPVI zu blockieren, verwendete ich ein Fab Fragment eines neuen anti-GPVI-Antikörpers, EMF-1. Andererseits schienen die Aktivierungsprozesse durch die Thrombinrezeptoren (Protease-aktivierende Rezeptoren 1 und 4) und durch Integrin αIIbβ3 bis in späteren Stadien der Thrombusbildung fortlaufend aktiv zu sein. Schließlich zeigten diese Arbeiten eine entscheidende Rolle des GPVI- und Syk-

abhängigen Signalwegs bei der Bildung von prokoagulatorischen Blutplättchen, die das negativ geladene Phospholipid Phosphatidylserin exponieren.

Kapitel 4 ist eine umfassende Studie zur Depletion von humanem GPVI (huGPVI) in Mäusen unter Verwendung transgener *hGP6$^{tg/tg}$* Mäuse. Zunächst zeige ich, dass die Behandlung dieser Tiere mit dem monoklonalen Antikörper JAQ1 zu einer partiellen Depletion von GPVI *in vivo* führt, wodurch Thrombozyten mit niedriger GPVI-Dichte (GPVI^{LO}) erzeugt werden, die nicht mehr auf die Liganden CRP oder Convulxin ansprechen. Das Blut JAQ1-behandelter Mäuse zeigte in Vollblut-Flusskammerstudien eine beeinträchtigte Bildung von Thrombozytenaggregaten auf Kollagen unter arteriellen Flussbedingungen. Zusätzliche Studien mit einem anderen monoklonalen anti-Maus-GPVI-Antikörper, JAQ4, in Kombination mit Wildtypmäusen zeigten, dass die Erzeugung des GPVI^{LO}-Thrombozytenphänotyps von Antikörpern mit niedriger Affinität abhängig ist. Die spezifischen und hochaffinen anti-huGPVI-Antikörper EMF-1 und EMF-2 waren der Lage, den Rezeptor in *hGP6$^{tg/tg}$*-Mäusen *in vivo* vollständig herunterzuregulieren. Die mit EMF-1 IgG behandelten hGP6$^{tg/tg}$ Mäuse waren in verschiedenen experimentellen Modellen stark gegen die Bildung von okklusiven arteriellen Thromben geschützt, während die Blutungszeiten bei Amputation der Schwanzspitze nur minimal beeinflusst wurden. Dies zeigt, dass die *in vivo* Herunterregulierung von huGPVI das Potenzial hat, eine sichere und wirksame Strategie zu werden, um die Thrombozytenaktivierung anzugreifen und thrombotische Erkrankungen zu behandeln.

Die in **Kapitel 5** vorgestellte Arbeiten beschreiben die erstmalige Charakterisierung einer CLEC-2 humanisierten Mauslinie (hCLEC-2^{KI}) und legt dar, dass diese bei der Beurteilung neuer Therapeutika gegen CLEC-2 hilfreich sein kann. Eingangs zeige ich, dass die transgenen Mäuse, die nur hCLEC-2 exprimieren, sich nicht von Wildtyp-Mäusen (mit murinen CLEC-2-Rezeptoren) in Bezug auf Blut-Lymphgefäß-Trennung

und Organentwicklung und -morphologie unterscheiden. Ebenso können die Thrombozyten von hCLEC-2^{KI} Mäusen vergleichbar wie Wildtyp-Thrombozyten aktiviert werden, sowie aggregieren und *spreaden*. Eine weitere wichtige Erkenntnis ist, dass der neuartige anti-hCLEC-2-Antikörper, HEL-1, hCLEC-2 *in vivo* vollständig herunterregulieren kann und dass diese Herunterregulierung keine Auswirkung auf die Hämostase hat. Andererseits zeigte sich, dass hCLEC-2^{KI} Tiere nach $FeCl_3$-induzierter Verletzung mesenterialer Arteriolen eine gestörte Thrombusbildung aufweisen - im Gegensatz zu Wildtyp-Mäusen, bei denen in allen getesteten Arteriolen ein vollständiger Gefäßverschluss beobachtet wurde. Paradoxerweise stellt die hCLEC-2-Depletion in hCLEC-2KI-Tieren einen Wildtyp-ähnlichen Phänotyp wieder her.

Abschließend diskutiere ich in **Kapitel 6** die Ergebnisse dieser Arbeit und vergleiche diese kritisch mit der aktuellen Literatur. Insbesondere betone ich die Relevanz und Bedeutung der Untersuchung und Erforschung verschiedener GPVI-*targeting* Strategien, die Möglichkeiten der Verwendung humanisierter Mausmodelle in der translationalen Forschung sowie die Implikationen und Herausforderungen zukünftiger therapeutischer Strategien, die auf CLEC-2 abzielen.

IMPACT

In conjunction with the coagulation cascade, platelets are key regulators of physiological hemostasis. However, at site of a ruptured or eroded atherosclerotic plaque, uncontrolled platelet activation can lead to the generation of vaso-occlusive thrombi, and to subsequent to life-threatening conditions such as heart attack and stroke. The gold standard for secondary arterial thrombosis prevention is a prophylactic treatment with antiplatelet ADP receptor antagonists and aspirin.[1-3] However, this treatment carries an inherent increase in the risk of bleeding.[4] To overcome this side effect, there is ongoing research to discover new therapeutic targets and antiplatelet drugs, which will allow better treatment of cardiovascular diseases with reduced side effects. In addition, platelet targeting may also be beneficial for the treatment of other pathological conditions such as deep vein thrombosis, inflammatory diseases (*e.g.*, acute respiratory distress syndrome, cancers and sepsis).[5-8]

Glycoprotein VI (GPVI) is the main collagen receptor on the platelet surface, which plays important roles in arterial thrombosis,[9] venous thrombosis,[10] cancer and sepsis.[6,11] On the other hand, its role in hemostasis seems to be dispensable, as suggested by the fact that patients deficient in GPVI do not display a severe bleeding disorder.[12] In this thesis, I investigated the role of GPVI in hemostasis and thrombosis *in vivo*, capitalizing on a mouse model with a human form of *GP6* (*hGP6*$^{tg/tg}$). In addition, I worked on the targeting of human GPVI *in vitro* and *in vivo,* thereby taking advantage of in-house generated antibodies. We tested the effect of the known anti-mouse GPVI antibody JAQ1 for the first time on human GPVI. This led to the discovery that JAQ1 binds to a structurally conserved epitope of mouse and human GPVI, which has been shifted in function during the evolution of the two species. In addition, the study gave proof of concept that the *hGP6*$^{tg/tg}$ mouse model is suitable for testing earlier developed and new anti-human GPVI compounds, as the model fully replicated the

effect of JAQ1 on human platelets (Chapter 2). The demonstration that JAQ1 binds to huGPVI in vitro and that the *hGP6*$^{tg/tg}$ model replicated what observed in human, served as foundation to the development of the pharmacological approach to generate huGPVI^{LO} platelets in vivo using JAQ1, as described in chapter 4.

In order to establish the time-dependent role of GPVI on whole-blood thrombus and clot formation, I modified a previously standardized microfluidic device, with a collagen and tissue factor surface. This allowed me to distinguish between early and late contributions of the thrombus-forming pathways. This effort revealed a crucial early role for GPVI-induced platelet signaling as well as for extrinsic coagulation-induced thrombin generation, which was confined to the first minutes of thrombus buildup. Markedly, my work indicate that this novel microfluidic application, represents a suitable in vitro approach to assess the contribution of different platelet and coagulation inhibitors/activators, and therefore improve pharmacokinetic studies of earlier developed as well as novel drugs for thrombosis and hemostasis.

To block GPVI, I employed a novel anti-GPVI Fab fragment, EMF-1, one clone out of 16 antibodies (EMF 1-16), with a strong GPVI-blocking effect (kindly provided by Emfret Analytics, Germany, on a collaborative basis). In addition, I found that the roles of platelet thrombin receptors and integrin αIIbβ3 were more prolongedly and extended throughout the whole process of thrombus buildup. Overall, this work revealed a more persistent contribution of thrombin-dependent platelet activation, whereas the initial collagen-dependent platelet activation was primarily important for platelet procoagulant activity and the promotion of fibrin deposition (Chapter 3).

In the following Chapter 4, I focused my attention on establishing the *in vivo* downregulation of huGPVI on mouse platelets. It appeared to be possible to partially down-regulate huGPVI from the surface of circulating mouse platelets using antibodies with a high on-off rate, thereby pharmacologically producing GPVI-low platelets, which

showed impaired responses to GPVI-specific agonists. In addition, I demonstrated that the complete depletion of GPVI with the EMF-1 antibody may be a safe, effective, long-term and reversible approach to target this platelet receptor. Finally, due to its long half-life and the low impact on hemostasis, I speculate that the scaled or complete depletion of GPVI may be a good therapeutic approach for the prevention of thrombotic events.

In the last Chapter 5, my work shifted towards the study of the related platelet receptor CLEC-2, also a member of the ITAM-signaling receptor family together with GPVI. We generated a mouse line humanized for CLEC-2 (hCLEC-2^{KI}), which proved to be suitable for the testing of anti-human CLEC-2 therapeutics *in vivo*. The study revealed that human CLEC-2 can fully compensate for mouse CLEC-2 during mouse development, and that hCLEC-2 has a minor role in hemostasis. On the other hand, it became clear that mouse CLEC-2 plays a more important role in thrombus stability than the human CLEC-2. Finally, this work generated useful tools to study the involvement of hCLEC-2 in different (patho)physiological conditions, which will help to better define a possible use of CLEC-2 targeting in therapeutic settings.

Overall, the thesis shows the current progress in the development of GPVI and CLEC-2 targeting strategies using humanized mouse lines. My work presented a modified microfluidic Maastricht flow-chamber as a useful tool for the detailed analysis of thrombus formation *in vitro*. Using the (novel) mouse lines with humanized GPVI and CLEC-2, we characterized several novel antibodies with interesting *in vivo* effects. Finally, this thesis reports for the first time a pre-clinical way of huGPVI depletion as a potential new therapeutic strategy. In this work, the results provide strong evidence that EMF-1 might be a novel promising lead for the generation of anti-GPVI drugs, with unprecedented high affinity and long half-life.

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Curriculum vitae

Curriculum Vitae

Stefano Navarro was born on November 18th 1993 in Naples, Italy. In 2015, he graduated in Health Biotechnology at the University of Naples Federico II. During the bachelor, he completed an internship at the Molecular Medicine and Medical Biotechnology Department of the Secondo Policlinico, Naples. After the bachelor, he graduated in Medical Biotechnologies at the University of Naples Federico II. During this period, he completed a 15 month internship at the Consiglio Nazionale delle Ricerche (CNR), where he focused on the study of molecular basis of a complex neuromuscular disorder (type II glycogen storage disease) using Whole Exome Sequencing. In 2018, he obtained the master degree with honor. In the same year, he started the PhD project as part of the joint doctoral Marie- Skłodowska-Curie programme (TAPAS) between the University of Würzburg (Germany) and the University of Maastricht (The Netherlands), under the joint supervision of Prof. Bernhard Nieswandt, Prof. Johan Heemskerk, Dr. Marijke Kuijpers and Dr. Heike Hermanns. During his PhD period, he physically worked at the Department of Biochemistry of the University of Maastricht and the Department of Experimental Biomedicine at the University of Würzburg. Stefano Navarro currently carries on research at the University of Würzburg under the supervision of Prof. Bernhard Nieswandt.

Würzburg, 20.01.2023

Publications

Full papers

1. Napolitano F, Bruno G, Terracciano C, Franzese G, Palomba NP, Scotto di Carlo F, Signoriello E, De Blasiis P, Navarro S, Gialluisi A, Melone MAB, Sampaolo S, Esposito T. Rare variants in autophagy and non-autophagy genes in late-onset Pompe disease: suggestions of their disease-modifying role in two Italian families. **Int. J. Mol. Sci.** 2021; 22:3625.

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3. Brown HC, Beck S, Navarro S, Di Y, Soriano Jerez EM, Kaczmarzyk J, Thomas SG, Mirakaj V, Watson SP, Nieswandt B, Stegner D. Antibody-mediated depletion of human CLEC-2 in a novel humanised mouse model. **Blood Adv**. 2023, in press.

4. Navarro S, Stegner D, Nieswandt B, Heemskerk JW, Kuijpers MJ. Temporal roles of platelet and coagulation pathways in collagen- and tissue factor-induced thrombus formation. **Int. J. Mol. Sci**. 2021; 23:358.

5. Navarro S, Starke A, Heemskerk JW, Kuijpers MJ, Stegner D, Nieswandt B. Targeting of a conserved epitope in mouse and human GPVI differently affects receptor function. **Int. J. Mol. Sci**. 2022; 23:8610.

Paper in preparation

1. Navarro S, Beck S, Stegner D, Nieswandt B. Scaled or complete in vivo depletion of human GPVI as a safe strategy for anti-thrombotic therapy.

Abstracts

1. Navarro S, Brown H, Stegner D, Nieswandt B, Kuijpers M. CRISPR-Cas9 based generation of a hGP6 mouse model as a tool for testing anti-GPVI agents. Poster presentation, Eureka symposium, Würzburg, Germany. 10.10.2019
2. Navarro S, Kuijpers MJ, Heemskerk JW, Stegner D, Nieswandt B. Targeting of a conserved epitope in mouse and human GPVI differently affects receptor function and in vivo depletion. Poster presentation, virtual ISTH conference, Milan, 11-15.07.2020
3. Navarro S, Beck S, Kuijpers MJ, Heemskerk JW, Stegner D, Nieswandt B. Characterization of a novel anti-GPVI antibody in a humanized GP6 mouse model. Oral presentation, ISTH conference, London, United Kingdom. 9-13.07.2022
4. Navarro S, Kuijpers MJ, Heemskerk JW, Stegner D, Nieswandt B. The antibody JAQ1 binds to both human and mouse GPVI but differently affect their function and in vivo depletion. Poster presentation, ISTH conference, London, United Kingdom. 9-13.07.2022
5. Navarro S, Stegner D, Nieswandt B, Heemskerk JW, Kuijpers MJ. Temporal roles of platelet and coagulation pathways in collagen and tissue factor induced thrombus formation. Poster presentation, ISTH conference, London, United Kingdom. 9-13.07.2022

Posters and oral communications

1. Navarro S. CRISPR-Cas9 based generation of a hGP6 mouse model as a tool for testing anti-GPVI agents. 14th International EUREKA Symposium GSLS. Oktober 2019, Würzburg, Germany. Poster presentation.

2. Navarro S. Targeting of a conserved epitope in mouse and human GPVI differently affects receptor function and in vivo depletion. ISTH 2020 Congress Milan, Italy (virtual). Poster.

3. Navarro S. Targeting of a conserved epitope in mouse and human GPVI differently affects receptor function and in vivo depletion. 4th Platelets Symposium of Collaborative Research Center TR 240, Würzburg, Germany. Poster presentation.

4. Navarro S. Characterization of a novel anti-GPVI antibody in a humanized GP6 mouse model. ISTH 2022 Congress London, United Kingdom. Oral presentation.

5. Navarro S. The antibody JAQ1 binds to both human and mouse GPVI but differently affect their function and in vivo depletion. ISTH 2022 Congress London, United Kingdom. Poster.

6. Navarro S. Temporal roles of platelet and coagulation pathways in collagen and tissue factor induced thrombus formation. ISTH 2022 Congress London, United Kingdom. Poster publications.

Dissertation Based on Several Published Manuscripts

Statement of individual author contributions and of legal second publication rights

Publication 1 (Chapter 2):

Targeting of a conserved epitope in mouse and human GPVI differently affects receptor function

Stefano Navarro[1,2], Andreas Starke[1], Johan W. M. Heemskerk[2,3], Marijke J. E. Kuijpers[2,4], David Stegner[1] and *Bernhard Nieswandt[1]

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Int. J. Mol. Sci. 2022, 23(15), 8610; https://doi.org/10.3390/ijms23158610

Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design Methods Development	BN	DS	JWMH, MJEK	SN	
Data Collection	SN	AS			
Data Analysis and Interpretation	SN	BN	DS	JWMH, MJEK	AS
Manuscript Writing Writing of Introduction Writing of Materials & Methods Writing of Discussion Writing of First Draft	 BN, SN BN, SN BN, SN SN	 DS DS DS DS	 JWMH JWMH JWMH	 MJEK MJEK MJEK	

Publication 2 (Chapter 3):

Temporal Roles of Platelet and Coagulation Pathways in Collagen- and Tissue Factor-Induced Thrombus Formation

Stefano Navarro[1,2,3], David Stegner[1,2], Bernhard Nieswandt[1,2], *Johan W.M. Heemskerk[3,4] and *Marijke J. E. Kuijpers[3,5]

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Int. J. Mol. Sci. 2022, 23(1), 358; https://doi.org/10.3390/ijms23010358

Participated in	**Author Initials,** Responsibility decreasing from left to right				
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Data Collection	SN				
Data Analysis and Interpretation	SN		JWMH, MJEK		
Manuscript Writing Writing of Introduction Writing of Materials & Methods Writing of Discussion Writing of First Draft	 JWMH, MJEK, SN JWMH, MJEK, SN JWMH, MJEK, SN SN		 BN BN BN	 DS DS DS	

Publication 3 (Chapter 4):

Scaled or complete in vivo depletion of human GPVI as a safe strategy for anti-thrombotic therapy

Short title: Anti-thrombotic potential of huGPVI depletion in vivo

Stefano Navarro[1, 2], Sarah Beck[1,2], David Stegner[1,2], Bernhard Nieswandt[1,2]

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Data Analysis and Interpretation	SN		BN	DS	SN
Manuscript Writing					
Writing of Introduction	SN	BN	SB	DS	SN
Writing of Materials & Methods	SN	BN	SB	DS	SN
Writing of Discussion	SN	BN	SB	DS	SN
Writing of First Draft	SN				SN

Publication 4 (Chapter 5):

Antibody-mediated depletion of human CLEC-2 in a novel humanised mouse model
Running title: hCLEC-2 depletion

Helena Brown[1, 2], Sarah Beck[1], Stefano Navarro[1,3], Ying Di[2], Eva María Soriano Jerez[1,4], Jana Kaczmarzyk[1], Steven G Thomas[2,5], Valbona Mirakaj[6], Steve P Watson[2,5], Bernhard Nieswandt[1], *David Stegner[1]

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August 2022, Blood Advances; DOI: 10.1182/bloodadvances.2021006463

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Data Collection	HB	DS, SB, SN	JK	EMSJ	
Data Analysis and Interpretation	HB	DS	SB, SN		
Manuscript Writing					
Writing of Introduction	DS	HB	BN, SPW	SGT, YD, VM	
Writing of Materials & Methods	DS	HB	BN, SPW	SGT, YD, VM	
Writing of Discussion	DS	HB	BN, SPW	SGT, YD, VM	
Writing of First Draft	HB	DS			

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Stefano Navarro	Würzburg, 20/01/2023	
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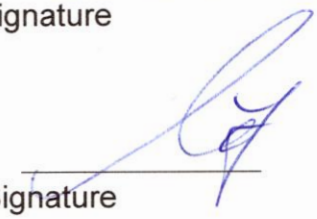
Bernhard Nieswandt	Würzburg, 20/01/2023	
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Statement of individual author contributions to figures/tables/chapters included in the Manuscripts

Publication 1 (Chapter 2):

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Int. J. Mol. Sci. 2022, 23(15), 8610; https://doi.org/10.3390/ijms23158610

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Table	**Author Initials,** Responsibility decreasing from left to right				
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Publication 2 (Chapter 3):

Temporal Roles of Platelet and Coagulation Pathways in Collagen- and Tissue Factor-Induced Thrombus Formation

Stefano Navarro[1,2,3], David Stegner[1,2], Bernhard Nieswandt[1,2], *Johan W.M. Heemskerk[3,4] and *Marijke J. E. Kuijpers[3,5]

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Int. J. Mol. Sci. 2022, 23(1), 358; https://doi.org/10.3390/ijms23010358

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2	SN			JWMH, MJEK	
3	SN			JWMH, MJEK	
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6	SN			JWMH, MJEK	
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Suppl. 4	SN			JWMH, MJEK	
Suppl. 5	SN			JWMH, MJEK	
Suppl. 6	SN			JWMH, MJEK	
Table	**Author Initials,** Responsibility decreasing from left to right				
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Publication 3 (Chapter 4):

Scaled or complete in vivo depletion of human GPVI as a safe strategy for anti-thrombotic therapy
Short title: Anti-thrombotic potential of huGPVI depletion in vivo

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6	SN				BN
Suppl. 1	SN				BN
Suppl. 2	SN				BN
Suppl. 3	SN				BN

Publication 4 (Chapter 5):

Antibody-mediated depletion of human CLEC-2 in a novel humanised mouse model
Running title: hCLEC-2 depletion

Helena Brown[1, 2], Sarah Beck[1], Stefano Navarro[1,3], Ying Di[2], Eva María Soriano Jerez[1,4], Jana Kaczmarzyk[1], Steven G Thomas[2,5], Valbona Mirakaj[6], Steve P Watson[2,5], Bernhard Nieswandt[1], *David Stegner[1]

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August 2022, Blood Advances; DOI: 10.1182/bloodadvances.2021006463

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Abstract	DS				
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2	HB	DS	SB		JK
Suppl. 1	HB		DS		
Suppl. 2	HB		DS		
Suppl. 3	HB		DS		
Suppl. 4	HB		DS	SN	
Suppl. 5	HB		DS	SB	
Suppl. 6	HB		DS		
Suppl. 7	HB		DS	SN	
Suppl. 8	HB		DS		
Suppl. 9	HB	DS	SB		
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I also confirm my primary supervisor´s acceptance.

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Affidavit

I hereby confirm that my thesis entitled "*Novel platelet glycoprotein VI and CLEC-2 targeting strategies: studies in humanized mouse models*" is the result of my own work. I did not receive any help or support from commercial consultants. All sources and/or materials applied are listed and specified in the thesis. Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Würzburg, 20/01/2023	
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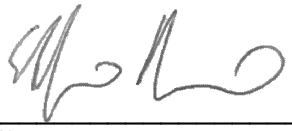
Eidestattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation *" Charakterisierung neuer Ansätze zur pharmakologischen Modulation der Thrombozytenrezeptoren GPVI und CLEC-2 in humanisierten Mausmodellen "* eigenständig, das heißt insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben. Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen wurde.

Würzburg, 20/01/2023	
Ort, Datum	*Unterschrift*

Acknowledgments

I would have not reached such an achievement if it weren´t for the help of all the great people that stood next to me during this years. The PhD has been a very stimulating and eventful process, which led me to an incredible academic and personal growth. Therefore, I would like to thank everyone that contributed to my work directly or indirectly even if not explicitly mentioned in this paragraph.

First, I would like to sincerely thank my supervisors, **Bernhard Nieswandt**, **Johan Heemskerk** and **Marijke Kuijpers**, for leading my way through all this, patiently helping me and correcting my mistakes and for making me see things from perspectives I had never thought before. If ever I will succeed, I will owe this to the marks left by the personal and professional values I have earned under your supervision.

Heike Hermanns, for your support, input and knowledge put into our meetings. **David Stegner**, for your constant support, robust assistance and ideas, for really helping me going through all this.

Steffi, **Ewa**, **Birgit**, **Juliana**, **Sylvie**, **Daniela**, for your irreplaceable technical assistance and hard work, your Job is highly appreciated. **Christine Mais**, for your work in managing the Lab, and the good company you gave me in the office in the last months of my PhD.

Katja Aurbach, above all the great time we spent together in and outside the office, I thank you for reaching out to me and offering me your friendship when I most needed it.

Isabelle Becker, for a real friendship and great professional support, the laughs we had together are countless. **Tobias Heib**, for the good time we spent together, for being a good friend, a good collegue. **Andreas Starke**, for standing and working next to me

the first months of my PhD. All member of the former office 3, **Bodo**, **Julia**, **Charly**, **Mariachiara**, **Lou**. The best office partners I could hope for.

Sarah Beck, for always being a valuable colleague, for your help and your most appreciated input into my work. **Zoltan Nagy**, for your friendship and support. **Timo Vögtle**, for your valuable help. All present and former members of the Nieswandt, Stegner, Schulze and Bender Lab.

A thank goes also to the people I met during my time in Maastricht, lovely people in a lovely city. All the members of the Maastricht platelet group, thank you for making it easy for me to adapt to a new Lab. **Marco Chavez**, for the quality time spent in our free time. **Claudia**, **Timo**, **Nicolò**, **Marco**, for making me love Bouldering.

I would like to thank all the people involved in the TAPAS consortium, and its coordinator **Steve Watson**, for giving me this unique opportunity and for going through this together.

Rabih, **Hannah**, **Jonny**, **Till**, **Ale**, **Angel**, I will thank you all together, for you are a group of dear friends everyone else should envy. You are all great, strong, bright people, which brought only good things into my life.

Francesco, all what we have been through together in the year and a half we shared the apartment is invaluable. Thank you for being next to me during the best and the worse moments and for helping me go through a part of my PhD. We went through the COVID pandemic together, sustaining each other, having good time every single day.

Ivan, I thank the lucky coincidence that made us meet both as colleagues and as friends at the same time. For our happy lunches and coffee breaks that made me go through most workdays in my last months. Also, because you are clearly the king of epitopes.

The other members of the Kroatengasse Family, **Feffo**, **Musti**, **Magnussino**, for the funniest and craziest time I have when we are together.

Johanna, for standing next to me, for giving me strength to go through this process, for the everlasting support and encouragement. Thank you for making me aware of my own value, for shedding a light where I can only see dark.

Stefano, my dearest friend, for having faced my worse and having accepted it, for being always next to me. For being the kind of person, I can always rely on 100% of the time, for being always good even in the most difficult of times. I have literally no memories of a life without you, since we have known each other for 27 years and I cannot believe how lucky I have been.

My mother, **Valeria**, for showing me the meaning of the word resilience, for pushing me through life no matter what, for believing in me in my darkest times, for only wanting my happiness, at all costs, always. For leaving me free, but never feel lonely. For giving the grownup one last chance to feel young and safe.

My sister, **Sabrina**, for being the example of the strong woman, before the concept was even a thing. You are undoubtedly one of the toughest people I know. Also, for showing me how life is taken by the neck, for teaching me that you can achieve anything, if you really want to.

All my family, **Luigi**, **Alessio**, **Ambra**, **Roberto**, **Rita**, **Emanuele**, **Luciano**, **Mena**, **Chiara**, **Mariagiulia**, **Maria**, for being always with me.

My incredible nonna, **Amalia**, for giving every bit of yourself for the family.

To my grandfather, **Antonio**, no longer amongst us, forever beside me. You showed me what it means to be a good man, I wish you could see, I am trying.